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(54) Title: ENZYMATIC MODIFICATION OF GLYCOPEPTIDES

(57) Abstract: The present invention provides glycoconjugates that are formed through the enzymatically-mediated coupling of a glycosyl moiety, e.g., on a peptide or lipid, and a modifying group that includes an acyl group. The conjugates include the modifying group tethered to the glycosyl moiety through a linking moiety that includes an acyl residue. Also provided are methods for preparing the conjugates of the invention.

ENZYMATIC MODIFICATION OF GLYCOPEPTIDES

CROSS-REFERENCES TO RELATED APPLICATIONS

5 [0001] The present application is related to U.S. Provisional Patent Application No. 60/611,790, filed September 20, 2004 and U.S. Provisional Patent Application No. 60/590,649, filed July 23, 2004; each of which are incorporated by reference in their entirety for all purposes.

BACKGROUND OF THE INVENTION

10 Field of the Invention

[0002] The present invention relates to conjugates formed between a glycosyl-containing species (e.g., glycopeptide, glycolipid) and a modifying group. The glycosyl-containing species and modifying group are linked through an enzymatically formed acyl-containing bond (e.g., amide, ester). The glycosyl-containing species are typically therapeutic agents.

15 Background

[0003] The administration of glycosylated and non-glycosylated therapeutic agents for engendering a particular physiological response is well known in the medicinal arts. For example, both purified and recombinant hGH are used for treating conditions and diseases due to hGH deficiency, *e.g.*, dwarfism in children, interferon has known antiviral activity and 20 granulocyte colony stimulating factor stimulates the production of white blood cells.

[0004] A principal factor that has limited the use of therapeutic peptides is the difficulty inherent in engineering an expression system to express a peptide having the glycosylation pattern of the wild-type peptide. Improperly or incompletely glycosylated peptides can be immunogenic; in a patient, an immunogenic response to an administered peptide can 25 neutralize the peptide and/or lead to the development of an allergic response in the patient. Other deficiencies of recombinantly produced glycopeptides include suboptimal potency and rapid clearance rates. The problems inherent in peptide therapeutics are recognized in the art, and various methods of eliminating the problems have been investigated.

[0005] Post-expression *in vitro* modification of peptides is an attractive strategy to remedy 30 the deficiencies of methods that rely on controlling glycosylation by engineering expression

systems; including both modification of glycan structures or introduction of glycans at novel sites. A comprehensive toolbox of recombinant eukaryotic glycosyltransferases is becoming available, making *in vitro* enzymatic synthesis of mammalian glycoconjugates with custom designed glycosylation patterns and glycosyl structures possible. See, for example, U.S. 5 Patent No. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and WO/9831826; US2003180835; and WO 03/031464.

[0006] Enzyme-based syntheses have the advantages of regioselectivity and stereoselectivity. Moreover, enzymatic syntheses are performed using unprotected substrates. Two principal classes of enzymes are used in the synthesis of carbohydrates, 10 glycosyltransferases (e.g., sialyltransferases, oligosaccharyltransferases, N-acetylglucosaminyltransferases), and glycosidases. The glycosidases are further classified as exoglycosidases (e.g., β -mannosidase, β -glucosidase), and endoglycosidases (e.g., Endo-A, Endo-M). Each of these classes of enzymes has been successfully used synthetically to prepare carbohydrates. For a general review, see, Crout *et al.*, *Curr. Opin. Chem. Biol.* **2**: 98-15 111 (1998).

[0007] Glycosyltransferases modify the oligosaccharide structures on glycopeptides. Glycosyltransferases are effective for producing specific products with good stereochemical and regiochemical control. Glycosyltransferases have been used to prepare oligosaccharides and to modify terminal N- and O-linked carbohydrate structures, particularly on 20 glycopeptides produced in mammalian cells. For example, the terminal oligosaccharides of glycopeptides have been completely sialylated and/or fucosylated to provide more consistent sugar structures, which improves glycopeptide pharmacodynamics and a variety of other biological properties. For example, β -1,4-galactosyltransferase was used to synthesize lactosamine, an illustration of the utility of glycosyltransferases in the synthesis of 25 carbohydrates (see, e.g., Wong *et al.*, *J. Org. Chem.* **47**: 5416-5418 (1982)). Moreover, numerous synthetic procedures have made use of α -sialyltransferases to transfer sialic acid from cytidine-5'-monophospho-N-acetylneuraminic acid to the 3-OH or 6-OH of galactose (see, e.g., Kevin *et al.*, *Chem. Eur. J.* **2**: 1359-1362 (1996)). Fucosyltransferases are used in synthetic pathways to transfer a fucose unit from guanosine-5'-diphosphofucose to a specific 30 hydroxyl of a saccharide acceptor. For example, Ichikawa prepared sialyl Lewis-X by a method that involves the fucosylation of sialylated lactosamine with a cloned fucosyltransferase (Ichikawa *et al.*, *J. Am. Chem. Soc.* **114**: 9283-9298 (1992)). For a discussion of recent advances in glycoconjugate synthesis for therapeutic use see, Koeller *et*

al., *Nature Biotechnology* **18**: 835-841 (2000). See also, U.S. Patent No. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and WO/9831826.

[0008] Glycosidases can also be used to prepare saccharides. Glycosidases normally catalyze the hydrolysis of a glycosidic bond. Under appropriate conditions, however, they 5 can be used to form this linkage. Most glycosidases used for carbohydrate synthesis are exoglycosidases; the glycosyl transfer occurs at the non-reducing terminus of the substrate. The glycosidase takes up a glycosyl donor in a glycosyl-enzyme intermediate that is either intercepted by water to give the hydrolysis product, or by an acceptor, to give a new 10 glycoside or oligosaccharide. An exemplary pathway using an exoglycosidase is the synthesis of the core trisaccharide of all N-linked glycopeptides, including the difficult β -mannoside linkage, which was formed by the action of β -mannosidase (Singh *et al.*, *Chem. Commun.* 993-994 (1996)).

[0009] In another exemplary application of the use of a glycosidase to form a glycosidic linkage, a mutant glycosidase was prepared in which the normal nucleophilic amino acid 15 within the active site is changed to a non-nucleophilic amino acid. The mutant enzymes do not hydrolyze glycosidic linkages, but can still form them. The mutant glycosidases are used to prepare oligosaccharides using an α -glycosyl fluoride donor and a glycoside acceptor molecule (Withers *et al.*, U.S. Patent No. 5,716,812). Although the mutant glycosidases are 20 useful for forming free oligosaccharides, it has yet to be demonstrated that such enzymes are capable of appending glycosyl donors onto glycosylated or non-glycosylated peptides, nor have these enzymes been used with unactivated glycosyl donors.

[0010] Although their use is less common than that of the exoglycosidases, 25 endoglycosidases are also utilized to prepare carbohydrates. Methods based on the use of endoglycosidases have the advantage that an oligosaccharide, rather than a monosaccharide, is transferred. Oligosaccharide fragments have been added to substrates using *endo*- β -N-acetylglucosamines such as *endo*-F, *endo*-M (Wang *et al.*, *Tetrahedron Lett.* **37**: 1975-1978); and Haneda *et al.*, *Carbohydr. Res.* **292**: 61-70 (1996)).

[0011] In addition to their use in preparing carbohydrates, the enzymes discussed above are 30 applied to the synthesis of glycopeptides. The synthesis of a homogenous glycoform of ribonuclease B has been published (Witte K. *et al.*, *J. Am. Chem. Soc.* **119**: 2114-2118 (1997)). The high mannose core of ribonuclease B was cleaved by treating the glycopeptide with endoglycosidase H. The cleavage occurred specifically between the two core GlcNAc

residues. The tetrasaccharide sialyl Lewis X was then enzymatically rebuilt on the remaining GlcNAc anchor site on the now homogenous protein by the sequential use of β -1,4-galactosyltransferase, α -2,3-sialyltransferase and α -1,3-fucosyltransferase V. Each enzymatically catalyzed step proceeded in excellent yield.

5 [0012] Methods combining both chemical and enzymatic synthetic elements are also known. For example, Yamamoto and coworkers (*Carbohydr. Res.* **305**: 415-422 (1998)) reported the chemoenzymatic synthesis of the glycopeptide, glycosylated Peptide T, using an endoglycosidase. The N-acetylglucosaminyl peptide was synthesized by purely chemical means. The peptide was subsequently enzymatically elaborated with the oligosaccharide of
10 human transferrin glycopeptide. The saccharide portion was added to the peptide by treating it with an endo- β -N-acetylglucosaminidase. The resulting glycosylated peptide was highly stable and resistant to proteolysis when compared to the peptide T and N-acetylglucosaminyl peptide T.

15 [0013] The use of glycosyltransferases to modify peptide structure with reporter groups has been explored. For example, Grossmer *et al.* (U.S. Patent No. 5,405,753) discloses the formation of a fluorescent-labeled cytidine monophosphate ("CMP") derivative of sialic acid and the use of the fluorescent glycoside in an assay for sialyl transferase activity and for the fluorescent labeling of cell surfaces, glycoproteins and gangliosides. Gross *et al.* (*Analyt. Biochem.* **186**: 127 (1990)) describe a similar assay. Bean *et al.* (U.S. Patent No. 5,432,059) discloses an assay for glycosylation deficiency disorders utilizing reglycosylation of a
20 deficiently glycosylated protein. The deficient protein is reglycosylated with a fluorescent-labeled CMP glycoside. Each of the fluorescent sialic acid derivatives is substituted with the fluorescent moiety at either the 9-position or at the amine that is normally acetylated in sialic acid. The methods using the fluorescent sialic acid derivatives are assays for the presence of
25 glycosyltransferases or for non-glycosylated or improperly glycosylated glycoproteins. The assays are conducted on small amounts of enzyme or glycoprotein in a sample of biological origin. The enzymatic derivatization of a glycosylated or non-glycosylated peptide on a preparative or industrial scale using a modified sialic acid has not been disclosed or suggested.

30 [0014] Considerable effort has also been directed towards the modification of cell surfaces by altering glycosyl residues presented by those surfaces. For example, Fukuda and coworkers have developed a method for attaching glycosides of defined structure onto cell

surfaces. The method exploits the relaxed substrate specificity of a fucosyltransferase that can transfer fucose and fucose analogs bearing diverse glycosyl substrates (Tsuboi *et al.*, *J. Biol. Chem.* **271**: 27213 (1996)).

[0015] The methods of modifying cell surfaces have not been applied in the absence of a cell to modify a glycosylated or non-glycosylated peptide. Moreover, the methods of cell surface modification are not utilized for the enzymatic incorporation preformed modified glycosyl donor moiety into a peptide. Moreover, none of the cell surface modification methods are practical for producing glycosyl-modified peptides on an industrial scale.

[0016] Enzymatic methods have also been used to activate glycosyl residues on a glycopeptide towards subsequent chemical elaboration. The glycosyl residues are typically activated using galactose oxidase, which converts a terminal galactose residue to the corresponding aldehyde. The aldehyde is subsequently coupled to an amine-containing modifying group. For example, Casares *et al.* (*Nature Biotech.* **19**: 142 (2001)) have attached doxorubicin to the oxidized galactose residues of a recombinant MHCII-peptide chimera.

[0017] In addition to manipulating the structure of glycosyl groups on polypeptides, interest has developed in preparing glycopeptides that are modified with one or more non-saccharide modifying group, such as water soluble polymers. Poly(ethyleneglycol) (“PEG”) is an exemplary polymer that has been conjugated to polypeptides. The use of PEG to derivatize peptide therapeutics has been demonstrated to reduce the immunogenicity of the peptides. For example, U.S. Pat. No. 4,179,337 (Davis *et al.*) discloses non-immunogenic polypeptides, such as enzymes and peptide hormones coupled to polyethylene glycol (PEG) or polypropylene glycol. Between 10 and 100 moles of polymer are used per mole polypeptide. Although the *in vivo* clearance time of the conjugate is prolonged relative to that of the polypeptide, only about 15% of the physiological activity is maintained. Thus, the prolonged circulation half-life is counterbalanced by the dramatic reduction in peptide potency.

[0018] The loss of peptide activity is directly attributable to the non-selective nature of the chemistries utilized to conjugate the water-soluble polymer. The principal mode of attachment of PEG, and its derivatives, to peptides is a non-specific bonding through a peptide amino acid residue. For example, U.S. Patent No. 4,088,538 discloses an enzymatically active polymer-enzyme conjugate of an enzyme covalently bound to PEG. Similarly, U.S. Patent No. 4,496,689 discloses a covalently attached complex of α -1

proteinase inhibitor with a polymer such as PEG or methoxypoly(ethyleneglycol) (“(m-) PEG”). Abuchowski *et al.* (*J. Biol. Chem.* **252**: 3578 (1977)) discloses the covalent attachment of (m-) PEG to an amine group of bovine serum albumin. U.S. Patent No. 4,414,147 discloses a method of rendering interferon less hydrophobic by conjugating it to an anhydride of a dicarboxylic acid, such as poly(ethylene succinic anhydride). PCT WO 5 87/00056 discloses conjugation of PEG and poly(oxyethylated) polyols to such proteins as interferon- β , interleukin-2 and immunotoxins. EP 154,316 discloses and claims chemically modified lymphokines, such as IL-2 containing PEG bonded directly to at least one primary amino group of the lymphokine. U.S. Patent No. 4,055,635 discloses pharmaceutical 10 compositions of a water-soluble complex of a proteolytic enzyme linked covalently to a polymeric substance such as a polysaccharide.

15 [0019] Another mode of attaching PEG to peptides is through the non-specific oxidation of glycosyl residues on a glycopeptide. The oxidized sugar is utilized as a locus for attaching a PEG moiety to the peptide. For example M’Timkulu (WO 94/05332) discloses the use of an amino-PEG to add PEG to a glycoprotein. The glycosyl moieties are randomly oxidized to 20 the corresponding aldehydes, which are subsequently coupled to the amino-PEG.

25 [0020] In each of the methods described above, poly(ethyleneglycol) is added in a random, non-specific manner to reactive residues on a peptide backbone. For the production of therapeutic peptides, it is clearly desirable to utilize a derivitization strategy that results in the formation of a specifically labeled, readily characterizable, essentially homogeneous product. A promising route to preparing specifically labeled peptides is through the use of enzymes, 20 such as glycosyltransferases to append a modified sugar moiety onto a peptide.

25 [0021] Glycosyl residues have also been modified to bear ketone groups. For example, Mahal and co-workers (*Science* **276**: 1125 (1997)) have prepared N-levulinoyl mannosamine (“ManLev”), which has a ketone functionality at the position normally occupied by the acetyl group in the natural substrate. Cells were treated with the ManLev, thereby incorporating a ketone group onto the cell surface. *See, also* Saxon *et al.*, *Science* **287**: 2007 (2000); Hang *et al.*, *J. Am. Chem. Soc.* **123**: 1242 (2001); Yarema *et al.*, *J. Biol. Chem.* **273**: 31168 (1998); and Charter *et al.*, *Glycobiology* **10**: 1049 (2000).

30 [0022] In addition to an industrially relevant method that utilizes the enzymatic conjugation to specifically conjugate a modified sugar to a peptide or glycopeptide, a method for

controlling and manipulating the position of glycosylation on a glycopeptide would be highly desirable.

[0023] Carbohydrates are attached to glycopeptides in several ways of which N-linked to asparagine and mucin-type O-linked to serine and threonine are the most relevant for recombinant glycoprotein therapeutics. A determining factor for initiation of glycosylation of a protein is the primary sequence context, although clearly other factors including protein region and conformation play roles. N-linked glycosylation occurs at the consensus sequence NXS/T, where X can be any amino acid but proline.

[0024] O-linked glycosylation is initiated by a family of about 20 homologous enzymes termed UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferases (GalNAc-transferases). O-linked glycosylation does not appear to be ruled by one simple consensus sequence, although studies of the GalNAc-transferase enzymes that initiate O-linked glycosylation clearly supports the notion that their acceptor specificities are driven by primary sequence contexts. Each of these enzymes transfer a single monosaccharide GalNAc to serine and threonine residues, but they transfer to different peptide sequences although they show a large degree of overlap in functions. It is envisioned that the substrate specificity of each GalNAc-transferase is ruled primarily by a linear short acceptor consensus sequence.

[0025] Recently, a method of producing an ester linked carbohydrate-peptide conjugate was described by Davis (WO 03/014371, published Feb. 20, 2003). In this publication, a vinyl ester amino acid group was reacted with a carbohydrate acyl acceptor in the presence of an enzyme such as a protease (such as a serine protease), lipase, esterase or acylase. At this time, however, no other substrates, e.g., glycopeptides, glycolipids, are known to conjugate with carbohydrate acyl acceptors under these conditions.

[0026] The present invention answers the need for modified therapeutic species in which a modified glycosyl moiety is conjugated onto N- or O-linked glycosylation sites of the peptides and other bioactive species, e.g., glycolipids, sphingosines, ceramides, etc. The invention provides a route to new therapeutic conjugates and addresses the need for more stable and therapeutically effective therapeutic species. Moreover, despite the efforts directed toward the enzymatic elaboration of saccharide structures, there remains still a need for alternative industrially practical methods for the modification of therapeutic agents, e.g., peptides, glycopeptides and lipids with modifying groups such as water-soluble polymers, therapeutic moieties, biomolecules and the like. Of particular interest are methods in which

the modified peptide has improved properties, which enhance its use as a therapeutic or diagnostic agent. The present invention fulfills these and other needs.

BRIEF SUMMARY OF THE INVENTION

[0027] Glycotherapeutics (e.g., glycopeptides, and glycolipids) present a challenging target for recombinant production of therapeutics. For example, carbohydrates are often indispensable for the function and favorable pharmacokinetic properties of glycopeptide therapeutics; however, many of the most robust expressions systems produce glycopeptides with non-human glycosylation patterns. Incorrect glycosylation can produce a peptide that is inactive, aggregated, antigenic and/or has unfavorable pharmacokinetics. Accordingly, considerable efforts are expended to develop recombinant expression cell systems capable of producing glycoproteins with biologically appropriate carbohydrate structures. This approach is hampered by numerous shortcomings, including cost, and heterogeneity and limitations in glycan structures.

[0028] Post-expression *in vitro* glyco-modification of glycotherapeutics, e.g., glycopeptides, is an attractive strategy to remedy the deficiencies of methods that rely on controlling glycosylation by engineering expression systems; including both modification of glycan structures or introduction of glycans at novel sites. A comprehensive toolbox of recombinant eukaryotic glycosyltransferases is becoming available, making *in vitro* enzymatic synthesis of mammalian glycoconjugates with custom designed glycosylation patterns and glycosyl structures possible. See, for example, U.S. Patent No. 5,876,980; 6,030,815; 5,728,554; 5,922,577; and WO/9831826; US2003180835; and WO 03/031464.

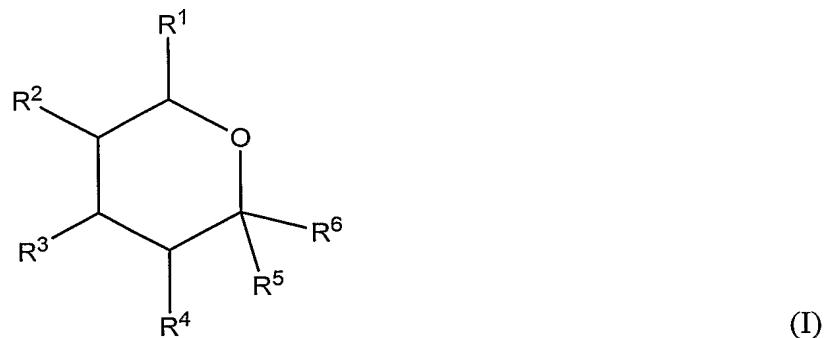
[0029] *In vitro* glycosylation offers a number of advantages compared to recombinant expression of glycoproteins of which custom design and higher degree of homogeneity of the glycosyl moiety are examples. Moreover, combining bacterial expression of glycotherapeutics with *in vitro* modification (or placement) of the glycosyl residue offers numerous advantages over traditional recombinant expression technology including reduced potential exposure to adventitious agents, increased homogeneity of product, and cost reduction.

[0030] Ideally, therapeutic conjugates of glycosyl-containing species, such as glycopeptides and glycolipids, are obtained using methods that provide the conjugates in a reproducible and predictable manner. Moreover, in forming the conjugates it is generally

preferred that the site of conjugation between the glycosyl-containing species and the modifying group is selected such that its modification does not adversely affect advantageous properties of glycosyl-containing species, e.g. activity, specificity, low antigenicity, low toxicity, etc.

5 [0031] The present invention provides an enzymatically-mediated method of forming conjugates between a glycosyl residue, amino acid residue (e.g., NH, OH, SH) or aglycone acid of a selected substrate (e.g., glycopeptide, glycolipid, etc.) and a modifying group, such as a water-soluble or water-insoluble polymer, a therapeutic moiety or a diagnostic agent. The invention exploits the recognition that certain classes of enzymes, which are typically degradative, can be made to run in a synthetic, rather than a degradative mode. Exemplary enzymes are those that are involved in the cleavage of bonds that include an acyl-containing component, such as an ester or an amide. Thus, enzymes of use in the present invention include, but are not limited to, proteases, lipases, acylases, and esterases. The invention can also be practiced with enzymes that are involved in the transfer of an acyl-containing moiety onto a substrate, e.g., acyltransferases, and amino acid t-RNA transferase.

10 [0032] In an exemplary aspect, the invention provides a lipid or peptide conjugate that includes a glycosyl residue having the formula:



20 in which the symbols R¹, R², R³, R⁴, and R⁵ independently represent H, OR^{7a}, N(R^{7a})₂, SR^{7a}, JC(O)R⁷, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl or substituted or unsubstituted heterocycloalkyl. The symbol J represents a bond, O, S or NH. The symbol R⁷ represents H, OR⁸, NR⁸R⁹, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, or substituted or unsubstituted heterocycloalkyl. Each R^{7a} is independently selected and represents H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl,

substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, or substituted or unsubstituted heterocycloalkyl.

[0033] R⁸ and R⁹ independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted

5 heteroaryl, or substituted or unsubstituted heterocycloalkyl.

[0034] In the conjugates of the invention, at least one of R¹, R², R³, R⁴, and R⁵ comprises a modifying group (e.g., polymer, therapeutic moiety, etc.) as discussed herein. The modifying group is linked to the glycosyl residue through a moiety that includes a carbonyl group, e.g., an acyl group (e.g., ROC(O)R'). In an exemplary embodiment, one of R⁸ or R⁹ is a water-

10 soluble polymer moiety (e.g., m-PEG, branched m-PEG).

[0035] The symbol R⁶ represents an amino acid residue of the peptide, a carbohydrate linker moiety covalently bound to an amino acid residue of the peptide, and combinations thereof.

[0036] Alternatively, when the conjugate includes a lipid, R⁶ represents an aglycone, a carbohydrate linker moiety covalently bound to an aglycone, and combinations thereof.

[0037] When R⁶ is a carbohydrate linker moiety, exemplary moieties bound to the glycosyl core shown in Formula I include Gal, GalNAc, Man, GlcNAc, Fuc, Sia, and Glu.

[0038] The invention also includes methods of preparing an acyl-modified glycosyl conjugate utilizing a mutant enzyme and mutant enzymes of use in the method. The mutant enzymes include a residue in the active site that is not found in the corresponding wild-type peptide. The residue acts to diminish or eliminate the hydrolytic activity of the enzyme. Art-recognized methods for preparing mutant peptides and screening them for a desired activity are of use in the present invention.

[0039] The invention also provides methods of improving pharmacological parameters of glycotherapeutics. For example, the invention provides a means for altering the pharmacokinetics, pharmacodynamics and bioavailability of glycosyl-containing therapeutics, e.g., cytokines, antibodies, growth hormones, enzymes, and glycolipids. In particular, the invention provides a method for lengthening the *in vivo* half-life of a glycotherapeutic by conjugating a water-soluble polymer to the therapeutic moiety through an acylated glycosyl linking group, e.g., an intact glycosyl linking group, or an acylated amino acid. In an exemplary embodiment, covalent attachment of polymers, such as

polyethylene glycol (PEG), e.g., m-PEG, to a therapeutic moiety affords conjugates having *in vivo* residence times, and pharmacokinetic and pharmacodynamic properties that are enhanced relative to the unconjugated therapeutic.

[0040] As discussed in the preceding section, art-recognized methods of covalent 5 PEGylation rely on non-selective chemical conjugation through reactive groups, typically amines, on amino acids or carbohydrates. A major shortcoming of chemical conjugation of PEG to proteins or glycoproteins is lack of selectivity, which often results in attachment of PEG at sites implicated in protein or glycoprotein bioactivity. Several strategies have been developed to address non-enzymatic site selective conjugation chemistries, however, one 10 universal method suitable for a variety of recombinant proteins has yet to be developed.

[0041] In contrast to art-recognized chemical conjugation methods, the present invention provides a novel, enzymatically-mediated strategy for selective conjugation, *e.g.*, PEGylation, directed to one or more specific location on a glycosyl residue of a glycopeptide or glycolipid. In an exemplary embodiment of the invention, site directed attachment of PEG 15 is provided by *in vitro* enzymatic acylation of specific residues on a glycosyl moiety by an activated PEG compound.

[0042] Additional aspects, advantages and objects of the present invention will be apparent from the detailed description that follows.

DESCRIPTION OF THE DRAWINGS

20 [0043] FIG. 1 is a table presenting exemplary sialyltransferases of use in the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations

[0044] Branched or un-branched PEG, poly(ethyleneglycol), including m-PEG, methoxy- 25 poly(ethylene glycol); branched or unbranched PPG, poly(propyleneglycol), including m-PPG, methoxy-poly(propylene glycol); Fuc, fucosyl; Gal, galactosyl; GalNAc, N-acetylgalactosaminyl; Glc, glucosyl; GlcNAc, N-acetylglucosaminyl; Man, mannosyl; ManAc, mannosaminyl acetate; Sia, sialic acid; and NeuAc, N-acetylneuraminy.

Definitions

[0045] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory 5 procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and hybridization are those well known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. The techniques and procedures are generally performed according to conventional methods in the art and various general references, which are provided throughout this document. The nomenclature used herein and 10 the laboratory procedures in analytical chemistry, and organic synthetic described below are those well known and commonly employed in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses.

[0046] The term “alkyl,” by itself or as part of another substituent, means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, 15 which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* C₁-C₁₀ means one to ten carbons). Examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n- 20 hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and isomers. The term “alkyl,” unless otherwise noted, is also meant to include those derivatives of alkyl 25 defined in more detail below, such as “heteroalkyl.” Alkyl groups, which are limited to hydrocarbon groups are termed “homoalkyl”.

[0047] The term “alkylene” by itself or as part of another substituent means a divalent radical derived from an alkane, as exemplified, but not limited, by -CH₂CH₂CH₂CH₂-*, and further includes those groups described below as “heteroalkylene.” Typically, an alkyl (or 30 alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the present invention. A “lower alkyl” or “lower alkylene” is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.*

[0048] The terms “alkoxy,” “alkylamino” and “alkylthio” (or thioalkoxy) are used in their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom, an amino group, or a sulfur atom, respectively.

[0049] The term “heteroalkyl,” by itself or in combination with another term, means, unless

5 otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, consisting of the stated number of carbon atoms and at least one heteroatom selected from the group consisting of O, N, Si and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and S and Si may be placed at any interior position of 10 the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, -CH₂-CH₂-O-CH₃, -CH₂-CH₂-NH-CH₃, -CH₂-CH₂-N(CH₃)-CH₃, -CH₂-S-CH₂-CH₃, -CH₂-CH₂-S(O)-CH₃, -CH₂-CH₂-S(O)₂-CH₃, -CH=CH-O-CH₃, -Si(CH₃)₃, -CH₂-CH=N-OCH₃, and -CH=CH-N(CH₃)-CH₃. Up to 15 two heteroatoms may be consecutive, such as, for example, -CH₂-NH-OCH₃ and -CH₂-O-Si(CH₃)₃. Similarly, the term “heteroalkylene” by itself or as part of another substituent means a divalent radical derived from heteroalkyl, as exemplified, but not limited by, -CH₂-CH₂-S-CH₂-CH₂- and -CH₂-S-CH₂-CH₂-NH-CH₂-.

For heteroalkylene groups, heteroatoms can also occupy either or both of the chain termini (e.g., alkyleneoxy, alkylenedioxy, alkyleneamino, alkylenediamino, and the like). Still further, for alkylene and heteroalkylene 20 linking groups, no orientation of the linking group is implied by the direction in which the formula of the linking group is written. For example, the formula -C(O)₂R'- represents both -C(O)₂R'- and -R'C(O)₂-.

[0050] In general, an “acyl substituent” is also selected from the group set forth above. As used herein, the term “acyl substituent” refers to groups attached to, and fulfilling the valence 25 of a carbonyl carbon that is either directly or indirectly attached to the polycyclic nucleus of the compounds of the present invention.

[0051] The terms “cycloalkyl” and “heterocycloalkyl”, by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of “alkyl” and “heteroalkyl”, respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the 30 position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include, but are not limited to, 1-(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-

morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1-piperazinyl, 2-piperazinyl, and the like.

[0052] The terms “halo” or “halogen,” by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally,

5 terms such as “haloalkyl,” are meant to include monohaloalkyl and polyhaloalkyl. For example, the term “halo(C₁-C₄)alkyl” is meant to include, but not be limited to, trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like.

[0053] The term “aryl” means, unless otherwise stated, a polyunsaturated, aromatic, hydrocarbon substituent which can be a single ring or multiple rings (preferably from 1 to 3

10 rings) which are fused together or linked covalently. The term “heteroaryl” refers to aryl groups (or rings) that contain from one to four heteroatoms selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl,

15 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-

20 benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxaliny, 5-quinoxaliny, 3-quinolyl, and 6-quinolyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below.

[0054] For brevity, the term “aryl” when used in combination with other terms (*e.g.*, aryloxy, arylthioxy, arylalkyl) includes both aryl and heteroaryl rings as defined above.

25 Thus, the term “arylalkyl” is meant to include those radicals in which an aryl group is attached to an alkyl group (*e.g.*, benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (*e.g.*, a methylene group) has been replaced by, for example, an oxygen atom (*e.g.*, phenoxyethyl, 2-pyridyloxymethyl, 3-(1-naphthoxy)propyl, and the like).

30 [0055] Each of the above terms (*e.g.*, “alkyl,” “heteroalkyl,” “aryl” and “heteroaryl”) include both substituted and unsubstituted forms of the indicated radical. Preferred substituents for each type of radical are provided below.

[0056] Substituents for the alkyl, and heteroalkyl radicals (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) are generally referred to as “alkyl substituents” and “heteroalkyl substituents,” respectively, and they can be one or more of a variety of groups selected from, but not limited to: -OR', =O, =NR', =N-OR', -NR'R'', -SR', -halogen, -SiR'R''R''', -OC(O)R', -C(O)R', -CO₂R', -CONR'R'', -OC(O)NR'R'', -NR''C(O)R', -NR'-C(O)NR''R''', -NR''C(O)₂R', -NR-C(NR'R''R''')=NR''', -NR-C(NR'R'')=NR''', -S(O)R', -S(O)₂R', -S(O)₂NR'R'', -NRSO₂R', -CN and -NO₂ in a number ranging from zero to (2m'+1), where m' is the total number of carbon atoms in such radical. R', R'', R''' and R'''' each preferably independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, e.g., aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R'', R''' and R'''' groups when more than one of these groups is present. When R' and R'' are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR'R'' is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term “alkyl” is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (e.g., -CF₃ and -CH₂CF₃) and acyl (e.g., -C(O)CH₃, -C(O)CF₃, -C(O)CH₂OCH₃, and the like).

[0057] Similar to the substituents described for the alkyl radical, the aryl substituents and heteroaryl substituents are generally referred to as “aryl substituents” and “heteroaryl substituents,” respectively and are varied and selected from, for example: halogen, -OR', =O, =NR', =N-OR', =NR'R'', -SR', -halogen, -SiR'R''R''', -OC(O)R', -C(O)R', -CO₂R', -CONR'R'', -OC(O)NR'R'', -NR''C(O)R', -NR'-C(O)NR''R''', -NR''C(O)₂R', -NR-C(NR'R'')=NR''', -S(O)R', -S(O)₂R', -S(O)₂NR'R'', -NRSO₂R', -CN and -NO₂, -R', -N₃, -CH(Ph)₂, fluoro(C₁-C₄)alkoxy, and fluoro(C₁-C₄)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where R', R'', R''' and R'''' are preferably independently selected from hydrogen, (C₁-C₈)alkyl and heteroalkyl, unsubstituted aryl and heteroaryl, (unsubstituted aryl)-(C₁-C₄)alkyl, and (unsubstituted aryl)oxy-(C₁-C₄)alkyl. When a compound of the invention includes more than one R group,

for example, each of the R groups is independently selected as are each R', R", R''' and R'''' groups when more than one of these groups is present.

[0058] Two of the aryl substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula $-T-C(O)-(CRR')_q-U-$, wherein T and

5 U are independently $-NR-$, $-O-$, $-CRR'$ - or a single bond, and q is an integer of from 0 to 3.

Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula $-A-(CH_2)_r-B-$, wherein A and B are independently $-CRR'$ - $, -O-$, $-NR-$, $-S-$, $-S(O)-$, $-S(O)_2-$, $-S(O)_2NR'$ - or a single bond, and r is an integer of from 1 to 4. One of the single bonds of the new ring so formed may optionally

10 be replaced with a double bond. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula $-(CRR')_s-X-(CR''R''')_d-$, where s and d are independently integers of from 0 to 3, and X is $-O-$, $-NR'$ - $, -S-$, $-S(O)-$, $-S(O)_2-$, or $-S(O)_2NR'$ - $. The substituents R, R', R'' and R''' are preferably$ independently selected from hydrogen or substituted or unsubstituted (C_1-C_6)alkyl.

15 **[0059]** As used herein, the term "heteroatom" includes oxygen (O), nitrogen (N), sulfur (S) and silicon (Si).

[0060] The term "nucleic acid" or "polynucleotide" refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known

20 analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated.

25 Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* **19**:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* **260**:2605-2608 (1985); and Rossolini *et al.*, *Mol. Cell. Probes* **8**:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA

30 encoded by a gene.

[0061] The term "gene" means the segment of DNA involved in producing a polypeptide chain. It may include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

[0062] The term “amino acid” refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ -5 carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical 10 structure as a naturally occurring amino acid. “Amino acid mimetics” refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0063] Amino acids may be referred to herein by either the commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical 15 Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0064] “Conservatively modified variants” applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, “conservatively modified variants” refers to those nucleic acids that encode identical or essentially identical amino acid 20 sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons 25 described without altering the encoded polypeptide. Such nucleic acid variations are “silent variations,” which are one species of conservatively modified variations. Every nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only 30 codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

[0065] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a “conservatively modified variant” where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[0066] As to amino acid sequences, one of skill will recognize that individual substitutions, 10 deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a “conservatively modified variant” where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies 15 homologs, and alleles of the invention.

[0067] The following eight groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Glycine (G);
- 20 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
- 25 7) Serine (S), Threonine (T); and
- 8) Cysteine (C), Methionine (M)

(see, e.g., Creighton, *Proteins* (1984)).

[0068] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical 30 Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0069] The term "mutating" or "mutation," as used in the context of altering the structure or enzymatic activity of a wild-type enzyme, refers to the deletion, insertion, or substitution of any nucleotide or amino acid residue, by chemical, enzymatic, or any other means, in a polynucleotide sequence encoding a that enzyme or the amino acid sequence of a wild-type 5 enzyme, respectively, such that the amino acid sequence of the resulting enzyme is altered at one or more amino acid residues. The site for such an activity-altering mutation may be located anywhere in the enzyme, but is preferably within the active site of the enzyme.

[0070] "Peptide" refers to a polymer in which the monomers are amino acids and are joined together through amide bonds, alternatively referred to as a polypeptide. Additionally, 10 unnatural amino acids, for example, β -alanine, phenylglycine and homoarginine are also included. Amino acids that are not gene-encoded may also be used in the present invention. Furthermore, amino acids that have been modified to include reactive groups, glycosylation sites, polymers, therapeutic moieties, biomolecules and the like may also be used in the 15 invention. All of the amino acids used in the present invention may be either the D - or L - isomer. The L -isomer is generally preferred. In addition, other peptidomimetics are also useful in the present invention. As used herein, "peptide" refers to both glycosylated and unglycosylated peptides. Also included are peptides that are incompletely glycosylated by a 20 system that expresses the peptide. For a general review, *see*, Spatola, A. F., in **CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES AND PROTEINS**, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983).

[0071] The term "peptide conjugate," refers to species of the invention in which a peptide is conjugated with an acyl-containing group that is attached to the peptide through a sugar residue.

[0072] The term "sialic acid" refers to any member of a family of nine-carbon carboxylated 25 sugars. The most common member of the sialic acid family is N-acetyl-neuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactonulopyranos-1-onic acid (often abbreviated as Neu5Ac, NeuAc, or NANA). A second member of the family is N-glycolyl-neuraminic acid (Neu5Gc or NeuGc), in which the N-acetyl group of NeuAc is hydroxylated. A third sialic acid family member is 2-keto-3-deoxy-noruloseonic acid (KDN) (Nadano *et al.* 30 (1986) *J. Biol. Chem.* **261**: 11550-11557; Kanamori *et al.*, *J. Biol. Chem.* **265**: 21811-21819 (1990)). Also included are 9-substituted sialic acids such as a 9-O-C₁-C₆ acyl-Neu5Ac like 9-O-lactyl-Neu5Ac or 9-O-acetyl-Neu5Ac, 9-deoxy-9-fluoro-Neu5Ac and 9-azido-9-deoxy-Neu5Ac. For review of the sialic acid family, *see*, e.g., Varki, *Glycobiology* **2**: 25-40 (1992);

Sialic Acids: Chemistry, Metabolism and Function, R. Schauer, Ed. (Springer-Verlag, New York (1992)). The synthesis and use of sialic acid compounds in a sialylation procedure is disclosed in international application WO 92/16640, published October 1, 1992.

[0073] As used herein, the term “modified sugar,” refers to a naturally- or non-naturally-

5 occurring carbohydrate of a glycosyl-containing compound to which an acyl-containing modifying group is added onto a glycosyl residue of a peptide in a process of the invention. The “modified sugar” is covalently functionalized with a “modifying group.” Useful modifying groups include, but are not limited to, water-soluble polymers, therapeutic moieties, diagnostic moieties, biomolecules and the like.

10 **[0074]** The term “water-soluble” refers to moieties that have a detectable degree of solubility in water. Methods to detect and/or quantify water solubility are well known in the art. Exemplary water-soluble polymers include peptides, saccharides, poly(ethers), poly(amines), poly(carboxylic acids) and the like. Peptides can have mixed sequences or be composed of a single amino acid, *e.g.*, poly(lysine), poly(aspartic acid), and poly(glutamic acid). An exemplary polysaccharide is poly(sialic acid). An exemplary poly(ether) is poly(ethylene glycol), *e.g.*, m-PEG. Poly(ethylene imine) is an exemplary polyamine, and poly(acrylic) acid is a representative poly(carboxylic acid).

15 **[0075]** The polymer backbone of the water-soluble polymer can be poly(ethylene glycol) (PEG). However, it should be understood that other related polymers are also suitable for use in the practice of this invention and that the use of the term PEG or poly(ethylene glycol) is intended to be inclusive and not exclusive in this respect. The term PEG includes poly(ethylene glycol) in any of its forms, including alkoxy PEG, alkyl PEG (*e.g.*, mPEG), difunctional PEG, multiarmed PEG, forked PEG, branched PEG, pendent PEG (*i.e.* PEG or related polymers having one or more functional groups pendent to the polymer backbone), or 20 PEG with degradable linkages therein.

25 **[0076]** The polymer backbone can be linear or branched. Branched polymer backbones are generally known in the art. Typically, a branched polymer has a central branch core moiety and a plurality of linear polymer chains linked to the central branch core. PEG is commonly used in branched forms that can be prepared by addition of ethylene oxide to various polyols, such as glycerol, pentaerythritol and sorbitol. The central branch moiety can also be derived from several amino acids, such as lysine. The branched poly(ethylene glycol) can be represented in general form as R(-PEG-OH)_m in which R represents the core moiety, such as

glycerol or pentaerythritol, and m represents the number of arms. Multi-armed PEG molecules, such as those described in U.S. Pat. No.s 5,932,462; 5,643,575; European Patent Application 0473,084 A2; WO 96/41813 (and its priority documents), can also be used as the polymer backbone.

5 [0077] Many other polymers are also suitable for the invention. Polymer backbones that are non-peptidic and water-soluble, with from 2 to about 300 termini, are particularly useful in the invention. Examples of suitable polymers include, but are not limited to, other poly(alkylene glycols), such as poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(oxyethylated polyol), poly(olefinic alcohol),
10 poly(vinylpyrrolidone), poly(hydroxypropylmethacrylamide), poly(α -hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazoline, poly(N-acryloylmorpholine), such as described in U.S. Pat. No. 5,629,384, which is incorporated by reference herein in its entirety, and copolymers, terpolymers, and mixtures thereof. Although the molecular weight of each chain of the polymer backbone can vary, it is typically in the range of from about 100 Da to
15 about 100,000 Da, often from about 6,000 Da to about 80,000 Da.

[0078] The terms "large-scale" and "industrial-scale" are used interchangeably and refer to a reaction cycle that produces at least about 250 mg, preferably at least about 500 mg, and more preferably at least about 1 gram of glycoconjugate at the completion of a single reaction cycle.

20 [0079] The term, "glycosyl linking group," as used herein refers to a glycosyl residue to which an acyl-containing modifying group (e.g., PEG moiety, therapeutic moiety, biomolecule) is covalently attached; the glycosyl linking group joins the modifying group to the remainder of the conjugate. In the methods of the invention, the "glycosyl linking group" is formed by the covalent modification, via an enzymatic acylation reaction of a glycosyl residue, thereby linking the agent to an amino acid and/or glycosyl residue on the peptide.
25 The glycosyl linking group can be a saccharide-derived structure that is degraded or degraded and modified prior to the addition of the modifying group (e.g., oxidation→Schiff base formation→reduction). Alternatively, the glycosyl linking group may be intact. An "intact glycosyl linking group" refers to a linking group that is derived from a glycosyl moiety in
30 which the saccharide monomer that links the modifying group and to the remainder of the conjugate is not degraded, e.g., oxidized, e.g., by sodium metaperiodate to create a locus of attachment for the modifying group. "Intact glycosyl linking groups" of the invention may

be derived from a naturally occurring oligosaccharide by addition of glycosyl unit(s) or removal of one or more glycosyl unit from a parent saccharide structure.

[0080] As used herein, the terms "polymer" and "polymers" are used interchangeably with the terms "oligomer" and "oligomers." The terms refer to species that have more than one

5 structurally related subunit, e.g., oligosaccharide and polysaccharide.

[0081] The term "targeting moiety," as used herein, refers to species that selectively localize in a particular tissue or region of the body. The localization is mediated by specific recognition of molecular determinants, molecular size of the targeting agent or conjugate, 10 ionic interactions, hydrophobic interactions and the like. Other mechanisms of targeting an

agent to a particular tissue or region are known to those of skill in the art. Exemplary 15 targeting moieties include antibodies, antibody fragments, transferrin, HS-glycoprotein, coagulation factors, serum proteins, β -glycoprotein, G-CSF, GM-CSF, M-CSF, EPO and the like.

[0082] As used herein, "therapeutic moiety" means any agent useful for therapy including, 15 but not limited to, antibiotics, anti-inflammatory agents, anti-tumor drugs, cytotoxins, and radioactive agents. "Therapeutic moiety" includes prodrugs of bioactive agents, constructs in which more than one therapeutic moiety is bound to a carrier, e.g, multivalent agents.

Therapeutic moiety also includes proteins and constructs that include proteins. Exemplary 20 proteins include, but are not limited to, Erythropoietin (EPO), Granulocyte Colony Stimulating Factor (GCSF), Granulocyte Macrophage Colony Stimulating Factor (GMCSF), Interferon (e.g., Interferon- α , - β , - γ), Interleukin (e.g., Interleukin II), serum proteins (e.g., Factors VII, VIIa, VIII, IX, and X), Human Chorionic Gonadotropin (HCG), Follicle Stimulating Hormone (FSH) and Lutenizing Hormone (LH) and antibody fusion proteins (e.g. Tumor Necrosis Factor Receptor ((TNFR)/Fc domain fusion protein)).

25 **[0083]** As used herein, "anti-tumor drug" means any agent useful to combat cancer including, but not limited to, cytotoxins and agents such as antimetabolites, alkylating agents, anthracyclines, antibiotics, antimitotic agents, procarbazine, hydroxyurea, asparaginase, corticosteroids, interferons and radioactive agents. Also encompassed within the scope of the term "anti-tumor drug," are conjugates of peptides with anti-tumor activity, e.g. TNF- α .
30 Conjugates include, but are not limited to those formed between a therapeutic protein and a glycoprotein of the invention. A representative conjugate is that formed between PSLG-1 and TNF- α .

[0084] As used herein, "a cytotoxin or cytotoxic agent" means any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracinedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Other toxins include, for example, ricin, CC-1065 and analogues, the duocarmycins. Still other toxins include diphtheria toxin, and snake venom (e.g., cobra venom).

[0085] As used herein, "a radioactive agent" includes any radioisotope that is effective in diagnosing or destroying a tumor. Examples include, but are not limited to, indium-111, cobalt-60. Additionally, naturally occurring radioactive elements such as uranium, radium, and thorium, which typically represent mixtures of radioisotopes, are suitable examples of a radioactive agent. The metal ions are typically chelated with an organic chelating moiety.

[0086] Many useful chelating groups, crown ethers, cryptands and the like are known in the art and can be incorporated into the compounds of the invention (e.g., EDTA, DTPA, DOTA, NTA, HDTA, *etc.* and their phosphonate analogs such as DTPP, EDTP, HDTp, NTP, *etc.*) See, for example, Pitt *et al.*, "The Design of Chelating Agents for the Treatment of Iron Overload," In, INORGANIC CHEMISTRY IN BIOLOGY AND MEDICINE; Martell, Ed.; American Chemical Society, Washington, D.C., 1980, pp. 279-312; Lindoy, THE CHEMISTRY OF MACROCYCLIC LIGAND COMPLEXES; Cambridge University Press, Cambridge, 1989; Dugas, BIOORGANIC CHEMISTRY; Springer-Verlag, New York, 1989, and references contained therein.

[0087] Additionally, a manifold of routes allowing the attachment of chelating agents, crown ethers and cyclodextrins to other molecules is available to those of skill in the art. See, for example, Meares *et al.*, "Properties of In Vivo Chelate-Tagged Proteins and Polypeptides." In, MODIFICATION OF PROTEINS: FOOD, NUTRITIONAL, AND PHARMACOLOGICAL ASPECTS;" Feeney, *et al.*, Eds., American Chemical Society, Washington, D.C., 1982, pp. 370-387; Kasina *et al.*, *Bioconjugate Chem.*, **9**: 108-117 (1998); Song *et al.*, *Bioconjugate Chem.*, **8**: 249-255 (1997).

[0088] As used herein, "pharmaceutically acceptable carrier" includes any material, which when combined with the conjugate retains the conjugates' activity and is non-reactive with the subject's immune systems. Examples include, but are not limited to, any of the standard

pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Other carriers may also include sterile solutions, tablets including coated tablets and capsules. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

5 [0089] As used herein, "administering" means oral administration, administration as a suppository, topical contact, intravenous, intraperitoneal, intramuscular, intralesional, or subcutaneous administration, administration by inhalation, or the implantation of a slow-release device, *e.g.*, a mini-osmotic pump, to the subject. Adminsitration is by any route including parenteral and transmucosal (*e.g.*, oral, nasal, vaginal, rectal, or transdermal), particularly by inhalation. Parenteral administration includes, *e.g.*, intravenous, 10 intramuscular, intra-arteriole, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial. Moreover, where injection is to treat a tumor, *e.g.*, induce apoptosis, administration may be directly to the tumor and/or into tissues surrounding the tumor. Other 15 modes of delivery include, but are not limited to, the use of liposomal formulations, intravenous infusion, transdermal patches, etc.

20 [0090] The term "isolated" refers to a material that is substantially or essentially free from components, which are used to produce the material. For peptide conjugates of the invention, the term "isolated" refers to material that is substantially or essentially free from components, which normally accompany the material in the mixture used to prepare the peptide conjugate. "Isolated" and "pure" are used interchangeably. Typically, isolated peptide conjugates of the 25 invention have a level of purity preferably expressed as a range. The lower end of the range of purity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

30 [0091] When the peptide conjugates are more than about 90% pure, their purities are also preferably expressed as a range. The lower end of the range of purity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% purity.

[0092] Purity is determined by any art-recognized method of analysis (*e.g.*, band intensity on a silver stained gel, polyacrylamide gel electrophoresis, HPLC, or a similar means).

[0093] “Essentially each member of the population,” as used herein, describes a characteristic of a population of peptide conjugates of the invention in which a selected percentage of the modified sugars added to a peptide are added to multiple, identical acceptor sites on the peptide. “Essentially each member of the population” speaks to the “homogeneity” of the sites on the peptide conjugated to a modified sugar and refers to conjugates of the invention, which are at least about 80%, preferably at least about 90% and more preferably at least about 95% homogenous.

[0094] “Homogeneity,” refers to the structural consistency across a population of acceptor moieties to which the modified sugars are conjugated. Thus, in a peptide conjugate of the invention in which each modified sugar moiety is conjugated to a site having the same structure as the site to which every other modified sugar is conjugated, the peptide conjugate is said to be about 100% homogeneous. Homogeneity is typically expressed as a range. The lower end of the range of homogeneity for the peptide conjugates is about 60%, about 70% or about 80% and the upper end of the range of purity is about 70%, about 80%, about 90% or more than about 90%.

[0095] When the peptide conjugates are more than or equal to about 90% homogeneous, their homogeneity is also preferably expressed as a range. The lower end of the range of homogeneity is about 90%, about 92%, about 94%, about 96% or about 98%. The upper end of the range of purity is about 92%, about 94%, about 96%, about 98% or about 100% homogeneity. The purity of the peptide conjugates is typically determined by one or more methods known to those of skill in the art, *e.g.*, liquid chromatography-mass spectrometry (LC-MS), matrix assisted laser desorption mass time of flight spectrometry (MALDITOF), capillary electrophoresis, and the like.

[0096] “Substantially uniform conjugate” or a “substantially uniform conjugation pattern,” when referring to a glycoconjugate species, refers to the percentage of glycosyl moieties to be acylated that are, in fact, acylated by a selected enzyme. A substantially uniform conjugation pattern exists if substantially all (as defined below) members of a glycosyl group population intended to be acylated are acylated.

[0097] The term “substantially” in the above definitions of “substantially uniform” generally means at least about 40%, at least about 70%, at least about 80%, or more

preferably at least about 90%, and still more preferably at least about 95% of the acceptor moieties for a particular glycosyltransferase are glycosylated.

Introduction

[0098] The present invention provides conjugates that bear sugars modified with one or

5 more acyl-containing moiety. The sugars can be attached to an amino acid or glycosyl residue of a peptide or glycopeptide, or onto a glycosyl residue of a glycolipid (e.g., sphingosine, ceramide, etc.). Also provided are enzymatically-mediated methods for producing the conjugates of the invention. The invention also provides pharmaceutical formulations that include a conjugate formed by a method of the invention.

10 **[0099]** The conjugates of the invention are formed between a therapeutic core molecule, e.g., glycopeptide, glycolipid, and diverse species such as water-soluble polymers, therapeutic moieties, diagnostic moieties, targeting moieties and the like. Also provided are conjugates that include two or more peptides linked together through a linker arm, *i.e.*, multifunctional conjugates. The multi-functional conjugates of the invention can include two
15 or more copies of the same peptide or a collection of diverse peptides with different structures and/or properties. In exemplary conjugates according to this embodiment, the linker between the two peptides is attached to at least one of the peptides through an acylated glycosyl linking group.

20 **[0100]** The conjugates of the invention are prepared by the enzymatic conjugation of an activated acyl-containing modifying group to a glycosyl residue, forming a ‘modified sugar’. When the conjugate of the invention is a glycopeptide conjugate, the modified sugar is attached directly to an amino acid of a glycosylation site, or to a glycosyl residue attached either directly or indirectly (e.g., through one or more glycosyl residue) to a glycosylation site.

25 **[0101]** The modified sugar, when interposed between the peptide (or glycosyl residue) and the modifying group on the sugar becomes what is referred to herein as a “glycosyl linking group,” *e.g.*, “an intact glycosyl linking group.” Using the exquisite selectivity of enzymes, such as proteases, lipases, esterases, acyltransferases, acylases and sugar amidases, the present method provides peptides that bear a desired group at one or more specific locations.
30 Thus, in exemplary conjugates according to the present invention, a modified sugar is attached directly to a selected locus on the peptide chain or, alternatively, the modified sugar is appended onto a carbohydrate moiety of a glycopeptide. Peptides in which modified

sugars are bound to both a glycopeptide carbohydrate and directly to an amino acid residue of the peptide backbone are also within the scope of the present invention.

[0102] The invention also provides a method for preparing a conjugate of the invention using an activated acyl-containing species. The method includes contacting a glycopeptide, 5 or glycolipid with an activated acyl-containing species and an enzyme for which the activated acyl species is a substrate, which transfers the acyl species onto the glycosyl residue of a peptide or glycolipid.

[0103] The methods of the invention, make it possible to assemble modified glycopeptides and glycolipids that have a substantially homogeneous derivatization pattern; the enzymes 10 used in the invention are generally selective for a particular glycosyl residue or for particular substituents, or substituent patterns, on a glycosyl residue. The methods are also practical for large-scale production of modified glycopeptide and glycolipid conjugates. In one embodiment the methods of the invention provide a practical means for large-scale preparation of glycopeptide and glycolipid conjugates having preselected uniform 15 derivatization patterns. The methods are particularly well suited for modification of therapeutic peptides, including but not limited to, glycopeptides that are incompletely glycosylated during production in cell culture cells (*e.g.*, mammalian cells, insect cells, plant cells, fungal cells, yeast cells, or prokaryotic cells) or transgenic plants or animals.

[0104] The methods of the invention also provide conjugates of glycosylated and 20 unglycosylated peptides, and glycolipids, with increased therapeutic half-life due to, for example, reduced clearance rate, or reduced rate of uptake by the immune or reticuloendothelial system (RES). Moreover, the methods of the invention provide a means for masking antigenic determinants on peptides, thus reducing or eliminating a host immune response against the peptide. Selective attachment of targeting agents to a peptide or 25 glycolipid using an appropriate modified sugar can also be used to target the peptide or glycolipid to a particular tissue or cell surface receptor that is specific for the particular targeting agent. Moreover, there is provided a class of peptides and glycolipids that are specifically modified with a therapeutic moiety conjugated through a glycosyl linking group.

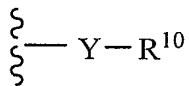
The Embodiments

Compositions

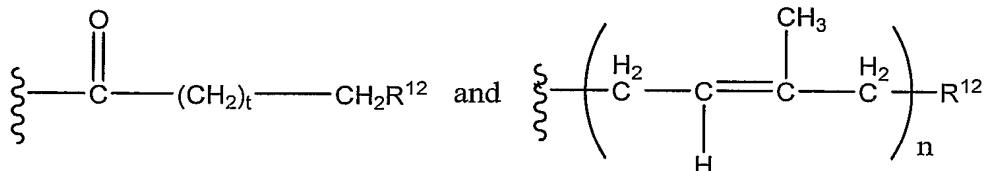
[0105] The present invention provides glyco-conjugates in which the sugar moiety is functionalized with a modifying group. The modifying group comprises an acyl group through which the modifying group is conjugated to the sugar moiety. The conjugation is typically achieved through the enzymatically-mediated reaction of an “activated modifying group” with an amine, sulfhydryl, primary hydroxyl, or secondary hydroxyl moiety on the sugar. In an exemplary embodiment, an amine moiety on the sugar is converted to an amide, a urethane or a urea through its reaction with the activated modifying group.

[0106] The present invention also provides conjugates in which the modifying group is covalently attached directly to the peptide or the lipid. The conjugation is typically achieved through the enzymatically-mediated reaction of an “activated modifying group” with an amine, sulfhydryl, primary hydroxyl, or secondary hydroxyl moiety on the peptide. In an exemplary embodiment, the “activated modifying groups” can be attached to a side chain of the peptide, such as the hydroxyl group of serine or threonine, the sulfur of cysteine, and/or the amine group of lysine. In an exemplary embodiment, an amine moiety on the peptide is converted to an amide, a urethane or a urea through its reaction with the activated modifying group.

[0107] The present invention also provides peptide conjugates in which the peptide is conjugated to a modifying group through a linking group comprising a lipid moiety. In one aspect, the invention provides a peptide conjugate comprising the moiety:



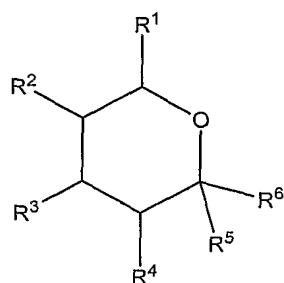
wherein Y is a member selected from O, S and NH, $\sim\sim\sim$ represents a connection to the remainder of the conjugate, and R^{10} is a member selected from:



n is an integer from 1 to 20; t is an integer from 1-20; $\sim\sim\sim$ represents a connection to Y, and R^{12} is a member selected from a water soluble polymer, a water insoluble polymer, a

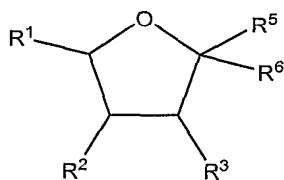
therapeutic moiety, and a diagnostic moiety. In an exemplary embodiment, R¹² is a water soluble polymer. Enzymes useful in the practice of the invention include but are not limited to, wild-type and mutant proteases, lipases, esterases, acylases, acyltransferases, glycosyltransferases, sulfotransferases, glycosidases, and the like. In some exemplary 5 embodiments, the enzymes may be wild-type or mutant prenyltransferases (e.g., farnesyltransferases, and geranylgeranyl transferases); N-myristoyltransferases, or palmitoyltransferases.

[0108] In an exemplary aspect, the invention provides a lipid or peptide conjugate that includes a glycosyl residue having a structure according to Formula I or Formula II:



10

(I)



(II)

in which the symbols R¹, R², R³, R⁴, R⁵ and R⁶ independently represent H, OR^{7a}, N(R^{7a})₂, SR^{7a}, JC(O)R⁷, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl or substituted or 15 unsubstituted heterocycloalkyl. The symbol J represents a bond, O, S or NH. The symbol R⁷ represents H, R⁸, OR⁸, NR⁸R⁹, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, or substituted or unsubstituted heterocycloalkyl. Each R^{7a} is independently selected and represents H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, 20 substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, or substituted or unsubstituted heterocycloalkyl.

[0109] R^8 and R^9 independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, or substituted or unsubstituted heterocycloalkyl.

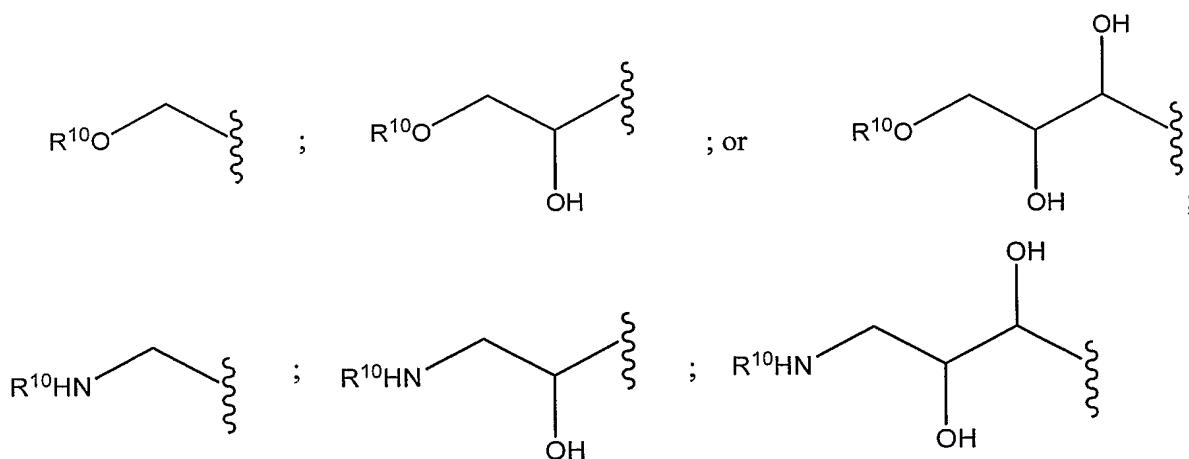
[0110] In the conjugates of the invention, at least one of R^1 , R^2 , R^3 , R^4 , R^5 and R^6 5 comprises a modifying group (e.g., polymer, water-soluble polymer, therapeutic moiety, etc.) as discussed herein. The modifying group is linked to the glycosyl residue through a moiety that includes a carbonyl group, e.g., an acyl group (e.g., $ROC(O)R'$). In an exemplary embodiment, one of R^8 or R^9 is a water-soluble polymer moiety (e.g., m-PEG, branched m-PEG).

[0111] The symbol R^6 represents an amino acid residue of the peptide, a carbohydrate linker moiety covalently bound to an amino acid residue of the peptide, and combinations thereof. 10

[0112] Alternatively, when the conjugate includes a lipid, R^6 represents an aglycone, a carbohydrate linker moiety covalently bound to an aglycone, and combinations thereof.

[0113] When R^6 is a carbohydrate linker moiety, exemplary moieties bound to the glycosyl core shown in Formula I include Gal, GalNAc, Man, GlcNAc, Fuc and Sia, and Glu. Those of skill will appreciate that the carbohydrate linker moiety can include these, and other, carbohydrate residues in essentially any combination and sequence. 15

[0114] In another exemplary embodiment, at least one of R^1 , R^2 , R^3 , R^4 , R^5 and R^6 is a 20 member selected from:

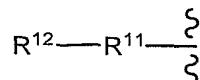


wherein $\sim\sim\sim$ represents a connection to the remainder of the conjugate, R^{10} includes a modifying group attached through a moiety that includes an acyl group. Exemplary

polymeric modifying groups include a poly(ether), a poly(sialic acid), and a poly(amino acid), e.g., poly(aspartic acid), poly(glutamic acid).

[0115] In a further exemplary embodiment, R^{10} can be H. In another exemplary embodiment, R^{10} can comprise a modifying group. In another exemplary embodiment, R^{10}

5 can have a structure according to the formula:

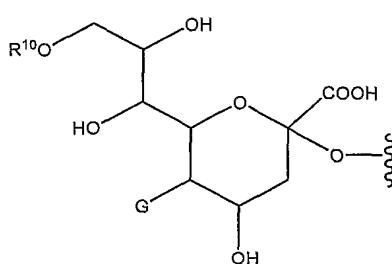


in which the symbol R^{11} represents a linker joining O to R^{12} or N to R^{12} and --- represents a connection to either the O or the N of the remainder of the conjugate. Exemplary linkers are members selected from substituted or unsubstituted alkyl and substituted or 10 unsubstituted heteroalkyl moieties. R^{12} is a modifying group.

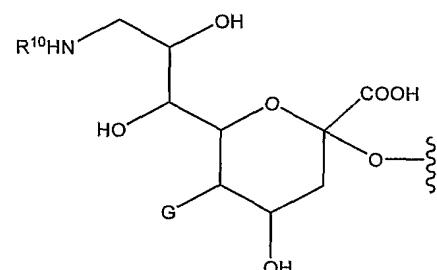
[0116] The linker is of any structure appropriate to join O and R^{12} or N and R^{12} with a level of stability appropriate for a selected application. In an exemplary embodiment in which R^{12} is a water-soluble polymer, e.g., m-PEG, the linker is a substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl moiety that has an acyl moiety attached, as a linking 15 moiety, to O or to N. An exemplary acyl-containing linking moiety is $-\text{C}(\text{O})\text{NH}$, affording a R^{11} moiety that is attached to the remainder of the saccharide through a urethane linkage.

Similarly, when the modifying group is a water-soluble polymer, the polymer can be joined to R^{11} through a linking moiety such as an amide or a urethane. The art-relevant for cross-linking two molecular species is well developed and it is within the abilities of one of skill in 20 the art to identify an appropriate R^{11} moiety and a precursor to this moiety.

[0117] In one embodiment, the present invention provides a conjugate comprising the moiety:



(IV) or

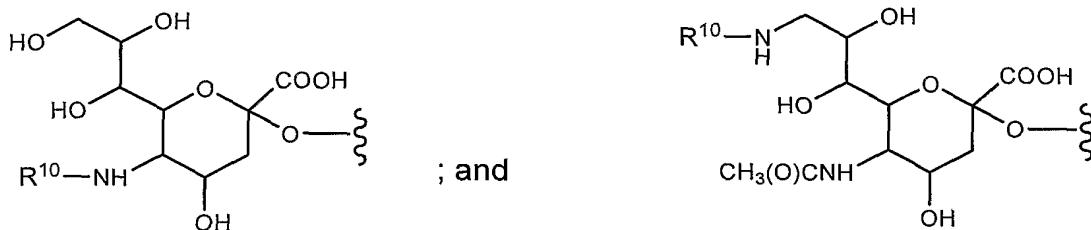


(V)

25 wherein --- represents a connection to the remainder of the conjugate, R^{10} is a member selected from H and R^{12} - R^{11} -; G is a member selected from HO, R^{12} - R^{11} -O-, NH₂ and R^{12} -

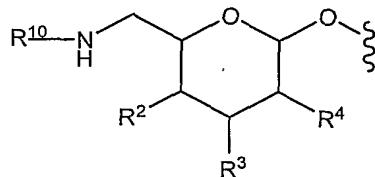
R¹¹-NH- and -C(O)(C₁-C₆)alkyl; R¹² is a modifying group, such as a straight-chain or branched poly(ethylene glycol) residue; and R¹¹ represents a linker joining O to R¹² or N to R¹², e.g., a bond (“zero order”), substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl. In exemplary embodiments, when the conjugate is according to 5 Formula (IV), R¹⁰ is H, G is R¹²-R¹¹-O-, R¹²-R¹¹-NH- and when G is -C(O)(C₁-C₆)alkyl, R¹⁰ is R¹²-R¹¹-.

[0118] In another exemplary embodiment, the invention provides a conjugate formed between a modified sugar of the invention and a lipid or peptide. In this embodiment, the sugar moiety of the modified sugar becomes a glycosyl linking group interposed between the 10 lipid or peptide substrate and the modifying group. An exemplary glycosyl linking group is an intact glycosyl linking group, in which the glycosyl moiety or moieties forming the linking group are not degraded by chemical (e.g., sodium metaperiodate) or enzymatic (e.g., oxidase) processes. Selected conjugates of the invention include a modifying group that is attached to the amine moiety of an amino-saccharide, e.g., mannosamine, glucosamine, galactosamine, 15 sialic acid etc. Exemplary modifying group-intact glycosyl linking group cassettes according to this motif are based on a sialic acid structure, such as those having the formulae:



[0119] In the formulae above, R¹⁰ is as described above. ~~~~ represents a connection to the remainder of the conjugate.

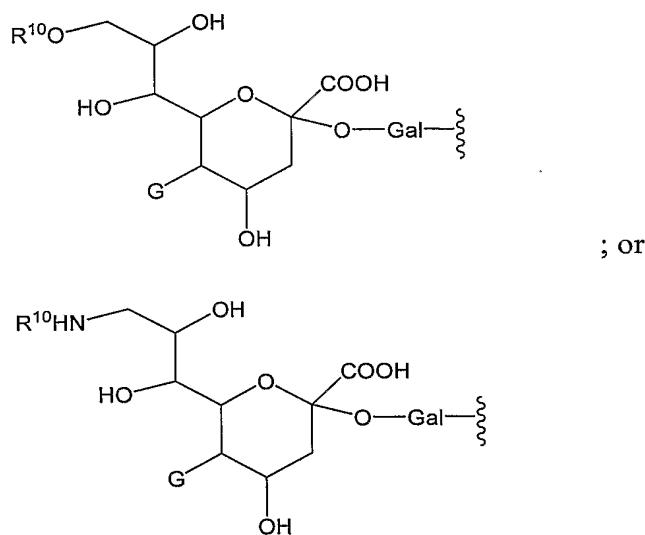
20 [0120] In still a further exemplary embodiment, the conjugate is formed between a lipid or peptide substrate and a glycosyl moiety in which the modifying group is attached through a linker at the 6-carbon position of the glycosyl moiety. Thus, illustrative conjugates according to this embodiment have the formula:



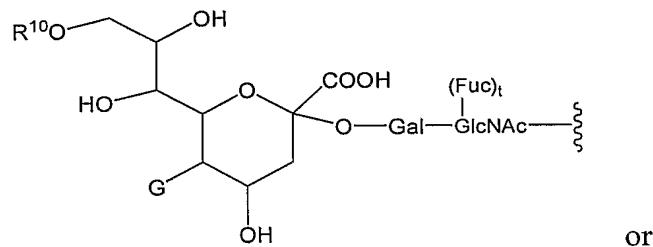
in which the radicals are as discussed above. Such glycosyl moieties include, without limitation, glucose, glucosamine, N-acetyl-glucosamine, galactose, galactosamine, N-acetyl-galactosamine, mannose, mannosamine, N-acetyl-mannosamine, and the like. $\sim\sim\sim$ represents a connection to the remainder of the conjugate.

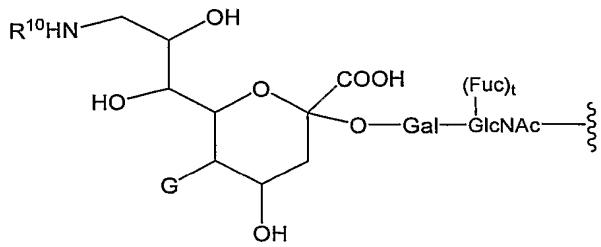
5 [0121] Due to the versatility of the methods available for modifying glycosyl residues on a therapeutic lipid or peptide, the glycosyl structures on the lipid or peptide conjugates of the invention can have substantially any structure. Moreover, the glycans can be O-linked or N-linked. As exemplified in the discussion below, each of the pyranose and furanose derivatives discussed above can be a component of a glycosyl moiety of a lipid or peptide.

10 [0122] The invention provides a modified lipid or peptide that includes a glycosyl group having the formula:



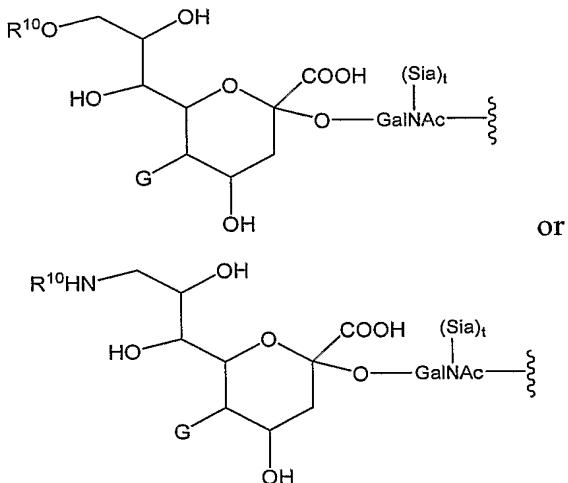
15 [0123] In other embodiments, the group has the formula:





in which the index t is 0 or 1.

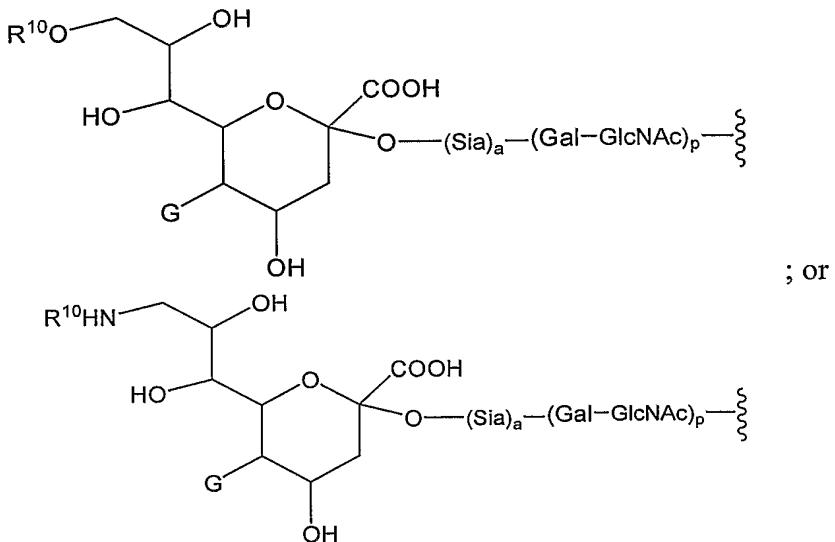
[0124] In a still further exemplary embodiment, the group has the formula:



5

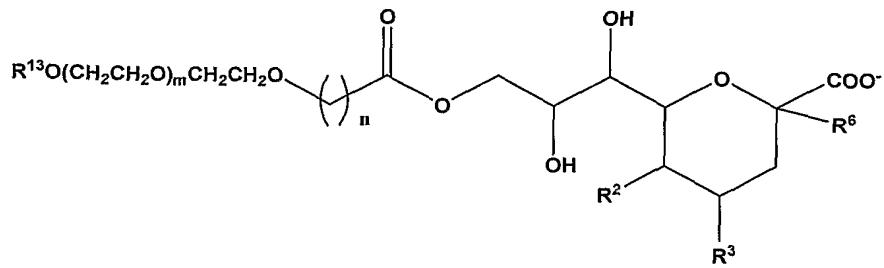
in which the index t is 0 or 1.

[0125] In yet another embodiment, the group has the formula:



10 in which the index p represents an integer from 1 to 10; and a is either 0 or 1.

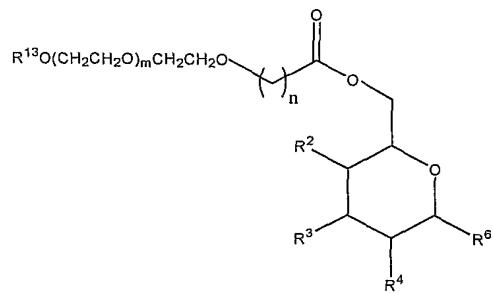
[0126] In an exemplary embodiment, the conjugate has a structure according to the following formula:



in which the index m is an integer from 1 to 2500, the index n is an integer from 0 to 40 and R¹³ is a member selected from H and substituted or unsubstituted alkyl.

[0127] In an exemplary embodiment, the conjugate has a structure according to the

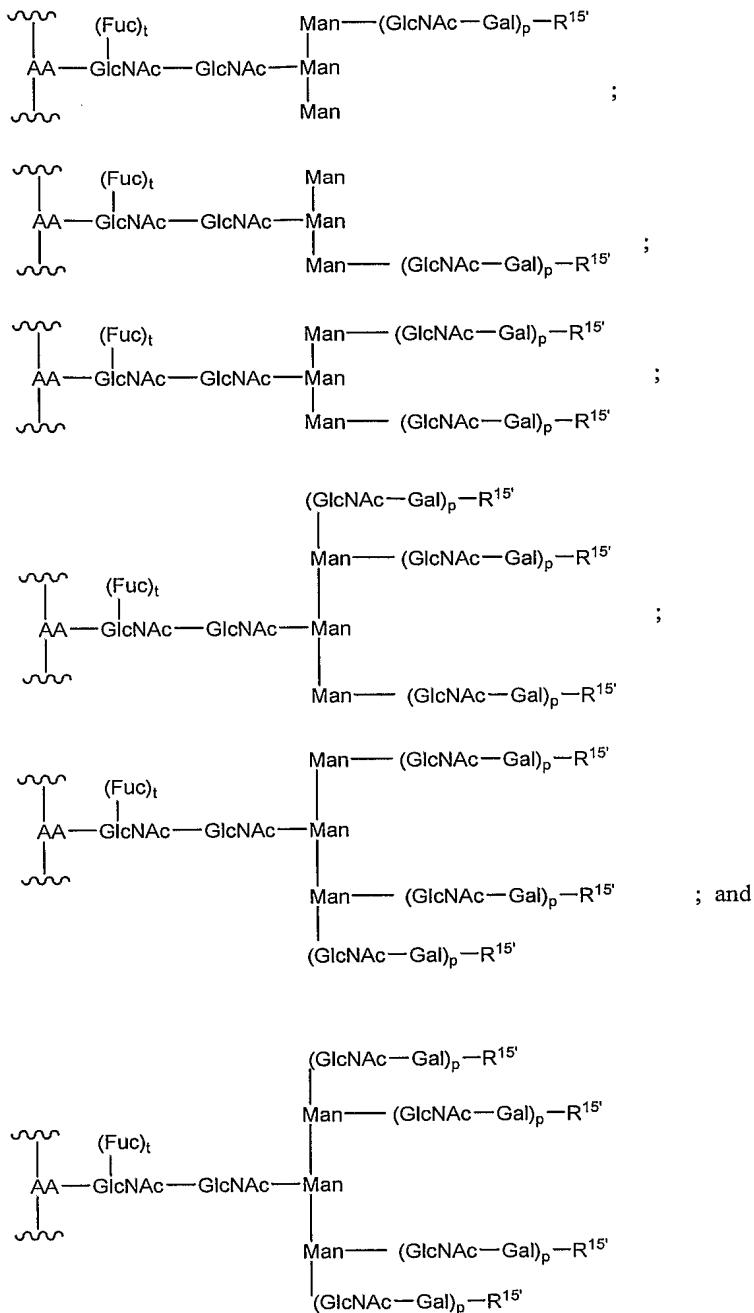
5 following formula:



in which the index m is an integer from 1 to 2500, the index n is an integer from 0 to 40; and R¹³ is a member selected from H and substituted or unsubstituted alkyl.

[0128] In an exemplary embodiment, a glycoPEGylated lipid or peptide conjugate of the

10 invention includes at least one N-linked glycosyl residue selected from the glycosyl residues set forth below:

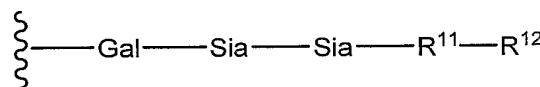


[0129] In the formulae above, the index t is 0 or 1 and the index p is an integer from 1 to 10, and 'AA' represents an amino acid of the peptide. $\sim\sim\sim$ represents a connection to the remainder of the peptide. The symbol $R^{15'}$ represents H, OH (e.g., Gal-OH), a sialyl moiety, a polymer modified sialyl moiety (i.e., glycosyl linking group-polymeric modifying moiety (Sia-L- $R^{15'}$)) or a sialyl moiety to which is bound a polymer modified sialyl moiety (e.g., Sia-Sia-L- $R^{15'}$) ("Sia-Sia^p"). Exemplary polymer modified glycosyl moieties have a structure according to Formulae I and II. An exemplary lipid or peptide conjugate of the invention will

include at least one glycan having a $R^{15'}$ that includes a structure according to Formulae I or II. The oxygen, with the open valence, of Formulae I and II is preferably attached through a glycosidic linkage to a carbon of a Gal or GalNAc moiety. In a further exemplary embodiment, the oxygen is attached to the carbon at position 3 of a galactose residue. In an exemplary embodiment, the modified sialic acid is linked α 2,3-to the galactose residue. In another exemplary embodiment, the sialic acid is linked α 2,6-to the galactose residue.

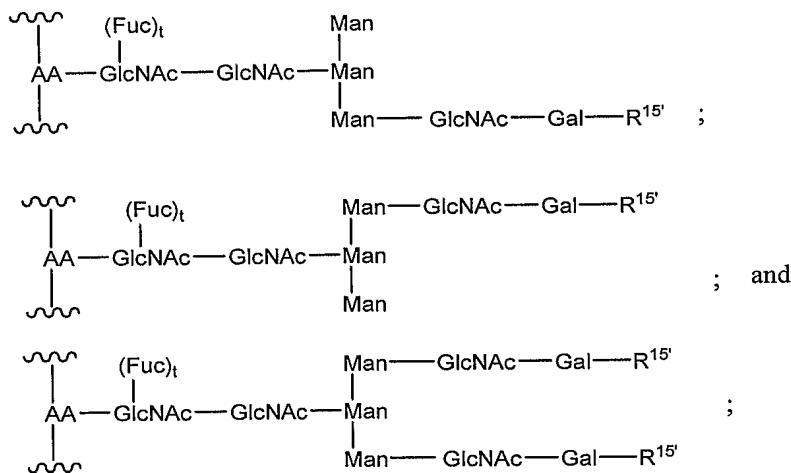
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[0130] In another exemplary embodiment, the invention provides a lipid or peptide conjugate that includes a glycosyl linking group, such as those set forth above, that is covalently attached to an amino acid residue of the peptide. In one embodiment according to 10 this motif, the glycosyl linking moiety is linked to a galactose residue through a Sia residue:



An exemplary species according to this motif is prepared by conjugating Sia- R^{11} - R^{12} to a terminal sialic acid of a glycan using an enzyme that forms Sia-Sia bonds, e.g., CST-II, ST8Sia-II, ST8Sia-III and ST8Sia-IV.

[0131] In another exemplary embodiment, the glycans have a formula that is selected 15 from the group:



and combinations thereof.

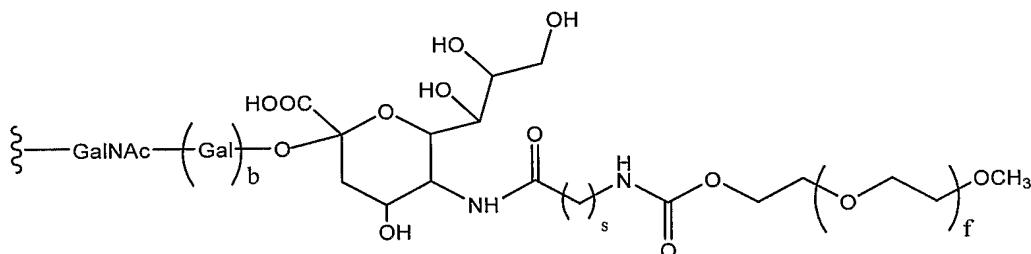
[0132] The glycans of this group generally correspond to those found on a lipid or peptide conjugate that is produced by insect (e.g., Sf-9) cells, following remodeling according to the 20 methods set forth herein. For example insect-derived lipid or peptide that is expressed with a

tri-mannosyl core is subsequently contacted with a GlcNAc donor and a GlcNAc transferase and a Gal donor and a Gal transferase. Appending GlcNAc and Gal to the tri-mannosyl core is accomplished in either two steps or a single step. A modified sialic acid is added to at least one branch of the glycosyl moiety as discussed herein. Those Gal moieties that are not 5 functionalized with the modified sialic acid are optionally “capped” by reaction with a sialic acid donor in the presence of a sialyl transferase.

10 [0133] In an exemplary embodiment, at least 60% of terminal Gal moieties in a population of peptides is capped with sialic acid, preferably at least 70%, more preferably, at least 80%, still more preferably at least 90% and even more preferably at least 95%, 96%, 97%, 98% or 99% are capped with sialic acid.

[0134] In each of the formulae above, R^{15'} is as discussed above. Moreover, an exemplary modified lipid or peptide of the invention will include at least one glycan with an R^{15'} moiety having a structure according to Formulae I or II.

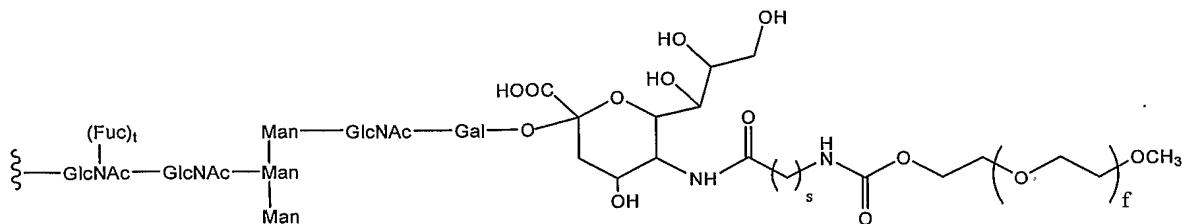
[0135] In an exemplary embodiment, the glycosyl linking moiety has the formula:



15

in which the index b is 0 or 1. ~~~~~ represents a connection to the remainder of the conjugate. The index s represents an integer from 1 to 10; and the index f represents an integer from 1 to 2500. Generally preferred is the use of a PEG moiety that has a molecular weight of about 20 kDa.

20 [0136] In another exemplary embodiment, the lipid or peptide is derived from insect cells, remodeled by adding GlcNAc and Gal to the mannose core and glycopegylated using a sialic acid bearing a linear PEG moiety, affording a lipid or peptide that comprises at least one moiety having the formula:



in which the index s represents an integer from 1 to 10; the index f represents an integer from 1 to 2500; and $\sim\sim\sim$ represents a connection to the remainder of the conjugate.

[0137] As discussed herein, R^{10} can comprise a linear or a branched modifying group, thus forming a linear or branched conjugate of the invention. An exemplary precursor of use to form the branched conjugates according to this embodiment of the invention has a structure according to Formulae IIIa or IIIb:



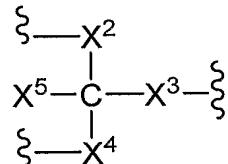
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[0138] The branched polymer species according to this formula are essentially pure water-soluble polymers. $X^{3'}$ is a moiety that includes an ionizable, e.g., OH, COOH, H_2PO_4 , HSO_3 , HPO_3 , and salts thereof, etc.) or other reactive functional group, e.g., *infra*. C is carbon. X^5 is preferably a non-reactive group (e.g., H, unsubstituted alkyl, unsubstituted heteroalkyl), and can be a polymeric arm. R^{16} and R^{17} are independently selected polymeric arms/modifying groups, e.g., nonpeptidic, nonreactive polymeric arms (e.g., PEG)). X^2 and X^4 are linkage fragments that are preferably essentially non-reactive under physiological conditions, which may be the same or different. An exemplary linker includes neither aromatic nor ester moieties. Alternatively, these linkages can include one or more moiety that is designed to degrade under physiologically relevant conditions, e.g., esters, disulfides, etc. X^2 and X^4 join polymeric arms R^{16} and R^{17} to C. When $X^{3'}$ is reacted with a reactive functional group of complementary reactivity on a linker, sugar or linker-sugar cassette, $X^{3'}$ is converted to a component of linkage fragment X^3 . R^{11} is as described above. Each R^{12} is independently selected as described above.

[0139] Exemplary linkage fragments for X^2 , X^3 and X^4 are independently selected and include S, SC(O)NH, HNC(O)S, SC(O)O, O, NH, NHC(O), (O)CNH and NHC(O)O, and OC(O)NH, CH₂S, CH₂O, CH₂CH₂O, CH₂CH₂S, (CH₂)_oO, (CH₂)_oS or (CH₂)_oY'-PEG wherein, Y' is S, NH, NHC(O), C(O)NH, NHC(O)O, OC(O)NH, or O and the index o is an integer from 1 to 50. In an exemplary embodiment, the linkage fragments X^2 and X^4 are different linkage fragments.

[0140] In an exemplary embodiment, the precursor (III), or an activated derivative thereof, is reacted with, and thereby bound to a sugar, an activated sugar or a sugar nucleotide through a reaction between X^3' and a group of complementary reactivity on the sugar moiety, e.g., an amine. Alternatively, X^3' reacts with a reactive functional group on a precursor to R^{11} . One or more of R^1 , R^2 , R^3 , R^4 , R^5 or R^6 of Formulae I and II can include the branched polymeric modifying moiety, or this moiety bound through R^{11} .

[0141] In an exemplary embodiment, the moiety:



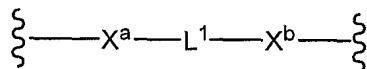
[0142] is R^{11} . In this embodiment, an exemplary linker is derived from a natural or unnatural amino acid, amino acid analogue or amino acid mimetic, or a small peptide formed from one or more such species. For example, certain branched polymers found in the compounds of the invention have the formula:



[0142] X^a is a linkage fragment that is formed by the reaction of a reactive functional group, e.g., X^3' , on a precursor of the branched polymeric modifying moiety and a reactive functional group on the sugar moiety, or a precursor to a linker. For example, when X^3' is a carboxylic acid, it can be activated and bound directly to an amine group pendent from an amino-saccharide (e.g., Sia, GalNH₂, GlcNH₂, ManNH₂, etc.), forming a X^a that is an amide. Additional exemplary reactive functional groups and activated precursors are described

hereinbelow. The index c represents an integer from 1 to 10. The other symbols have the same identity as those discussed above.

[0143] In another exemplary embodiment, X^a is a linking moiety formed with another linker:



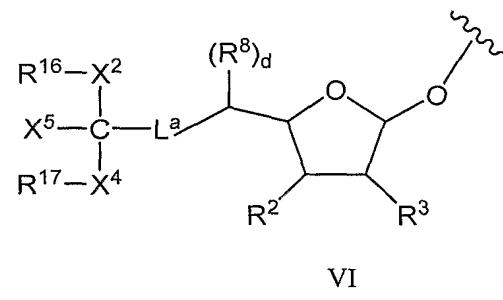
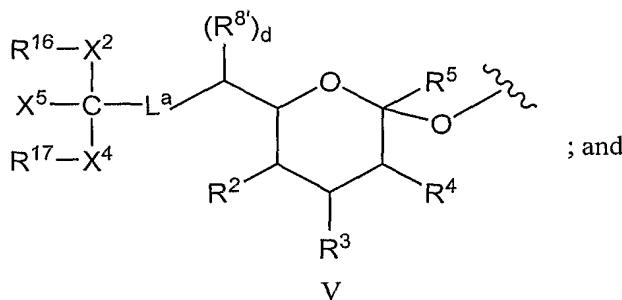
in which X^b is a second linkage fragment and is independently selected from those groups set forth for X^a , and, similar to R^{11} , L^1 is a bond, substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl.

[0144] Exemplary species for X^a and X^b include S, $SC(O)NH$, $HNC(O)S$, $SC(O)O$, O, NH, 10 NHC(O), $C(O)NH$ and $NHC(O)O$, and $OC(O)NH$.

[0145] In another exemplary embodiment, X^4 is a peptide bond to R^{17} , which is an amino acid, di-peptide (e.g., Lys-Lys) or tri-peptide (E.G., Lys-Lys-Lys) in which the alpha-amine moiety(ies) and/or side chain heteroatom(s) are modified with a polymeric modifying moiety.

[0146] In a further exemplary embodiment, the conjugates of the invention include a

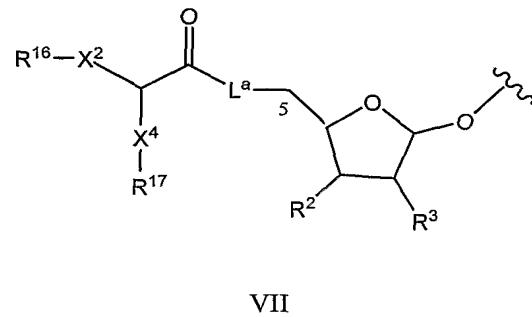
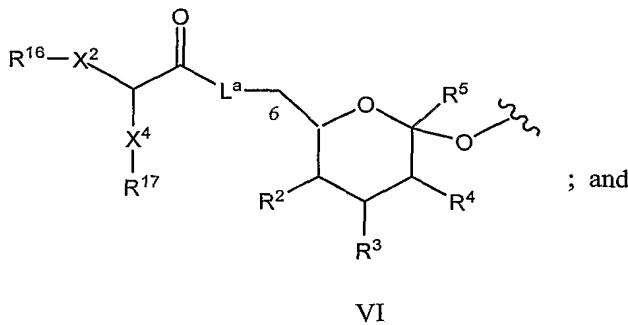
15 moiety, e.g., an $R^{15'}$ moiety that has a formula that is selected from:



in which the identity of the radicals represented by the various symbols is the same as that discussed hereinabove. L^a is a bond or a linker as discussed above for R^{11} and L^1 , e.g., substituted or unsubstituted alkyl or substituted or unsubstituted heteroalkyl moiety. In an

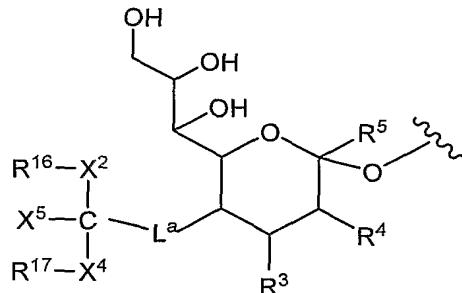
20 exemplary embodiment, L^a is a moiety of the side chain of sialic acid that is functionalized with the polymeric modifying moiety as shown. Exemplary L^a moieties include substituted or unsubstituted alkyl chains that include one or more OH or NH_2 .

[0147] In yet another exemplary embodiment, the invention provides conjugates having a moiety, e.g., an $R^{15'}$ moiety with formula:



The identity of the radicals represented by the various symbols is the same as that discussed hereinabove. As those of skill will appreciate, the linker arm in Formulae VI and VII is equally applicable to other modified sugars set forth herein. In exemplary embodiment, the species of Formulae VI and VII are the R¹⁵ moieties attached to the glycan structures set forth herein.

[0148] In yet another exemplary embodiment, the lipid or peptide includes an R¹⁵ moiety with the formula:



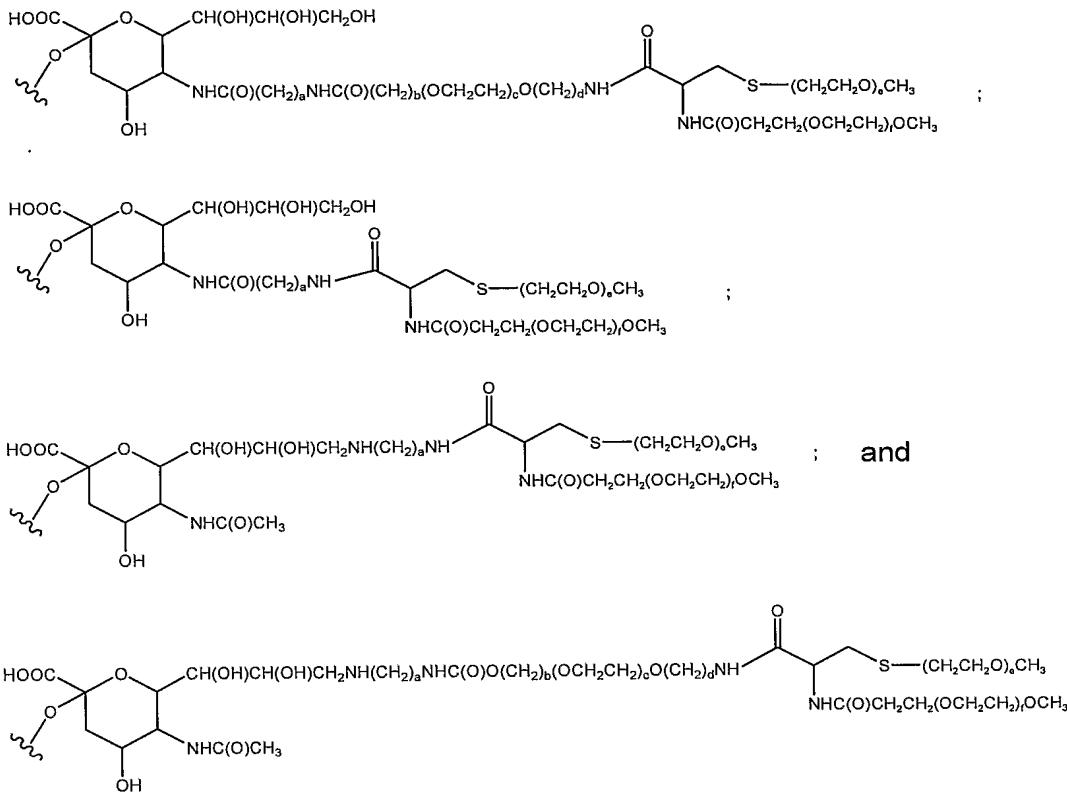
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in which the identities of the radicals are as discussed above. An exemplary species for L^a is -(CH₂)_jC(O)NH(CH₂)_hC(O)NH-, in which h and j are independently selected integers from 0 to 10. A further exemplary species is -C(O)NH-.

[0149] The embodiments of the invention set forth above are further exemplified by reference to species in which the polymer is a water-soluble polymer, particularly poly(ethylene glycol) ("PEG"), e.g., methoxy-poly(ethylene glycol). Those of skill will appreciate that the focus in the sections that follow is for clarity of illustration and the various motifs set forth using PEG as an exemplary polymer are equally applicable to species in which a polymer other than PEG is utilized.

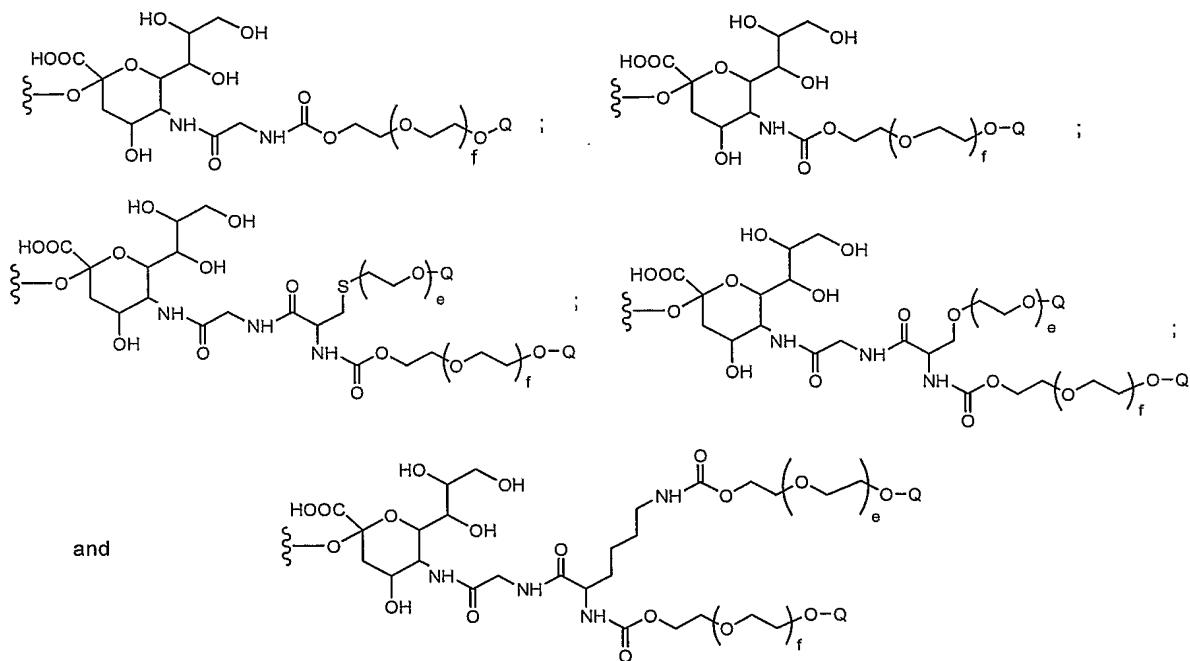
[0150] PEG of any molecular weight, e.g., 1 kDa, 2 kDa, 5 kDa, 10 kDa, 15 kDa, 20 kDa, 30 kDa and 40 kDa is of use in the present invention.

[0151] In an exemplary embodiment, the $R^{15'}$ moiety has a formula that is a member selected from the group:



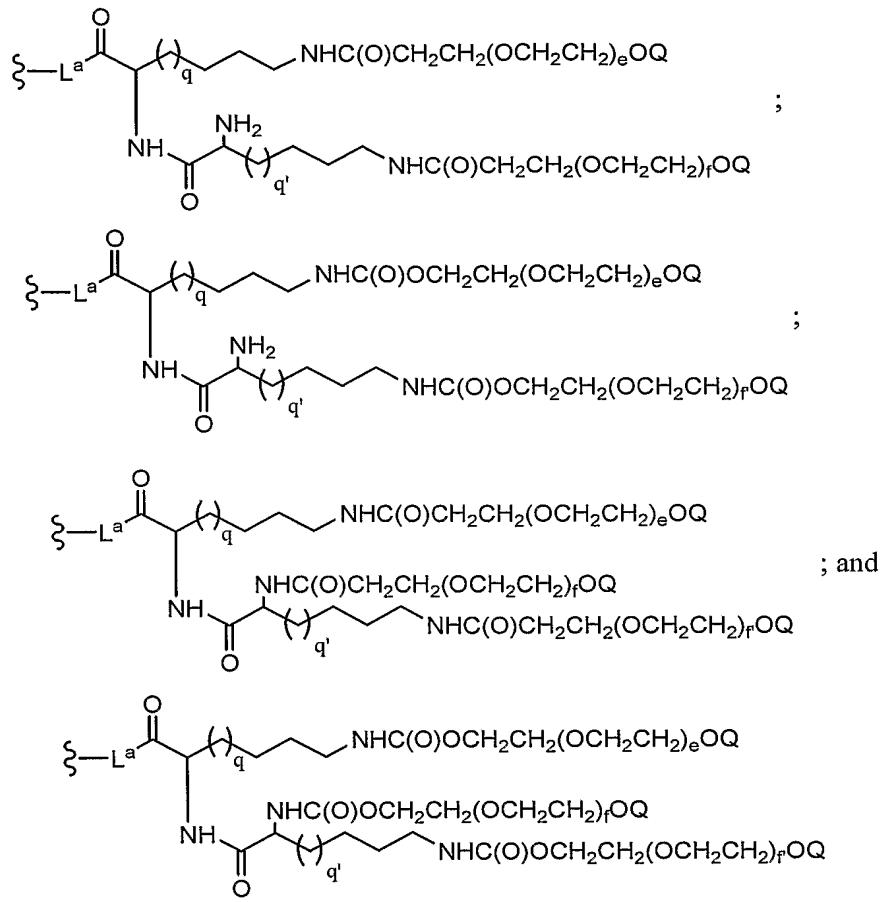
In each of the structures above, the linker fragment $-NH(CH_2)_a-$ can be present or absent.

5 **[0152]** In other exemplary embodiments, the lipid or peptide conjugate includes an $R^{15'}$ moiety selected from the group:

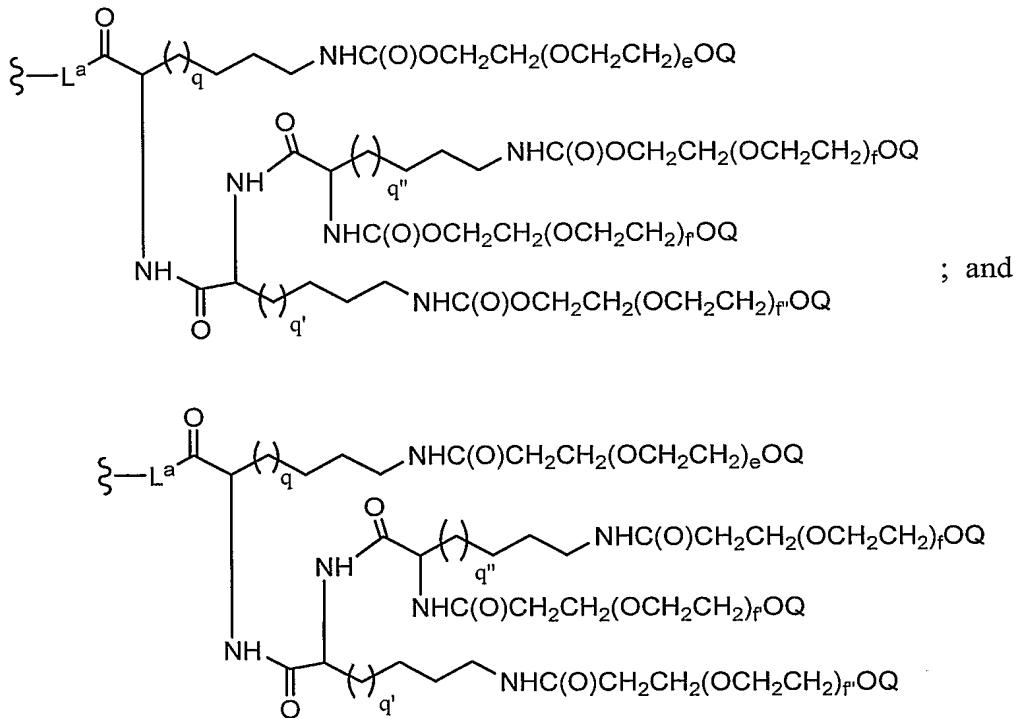


[0153] In each of the formulae above, the indices e and f are independently selected from the integers from 1 to 2500. In further exemplary embodiments, e and f are selected to provide a PEG moiety that is about 1 kD, 2 kD, 10 kD, 15 kD, 20 kD, 30 kD or 40 kD. The symbol Q represents substituted or unsubstituted alkyl (e.g., C₁-C₆ alkyl, e.g., methyl), substituted or unsubstituted heteroalkyl or H.

[0154] Other branched polymers have structures based on di-lysine (Lys-Lys) peptides, e.g.:



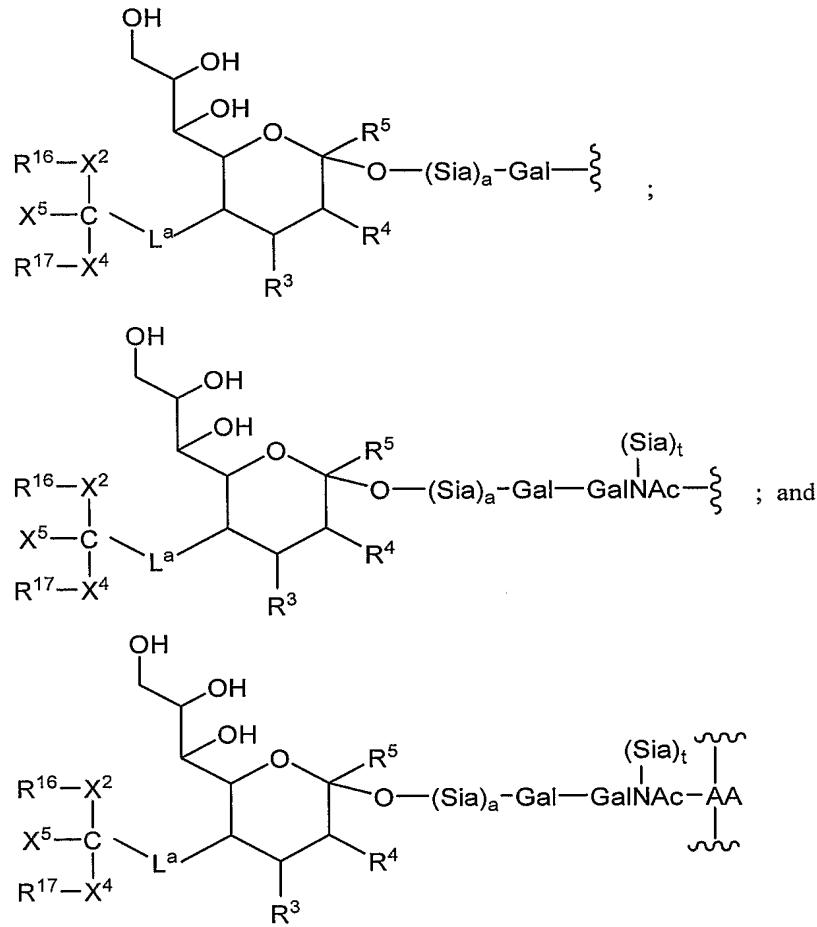
and tri-lysine peptides (Lys-Lys-Lys), e.g.:



In each of the figures above, the indices e, f, f' and f'' represent integers independently selected from 1 to 2500. The indices q, q' and q'' represent integers independently selected from 1 to 20.

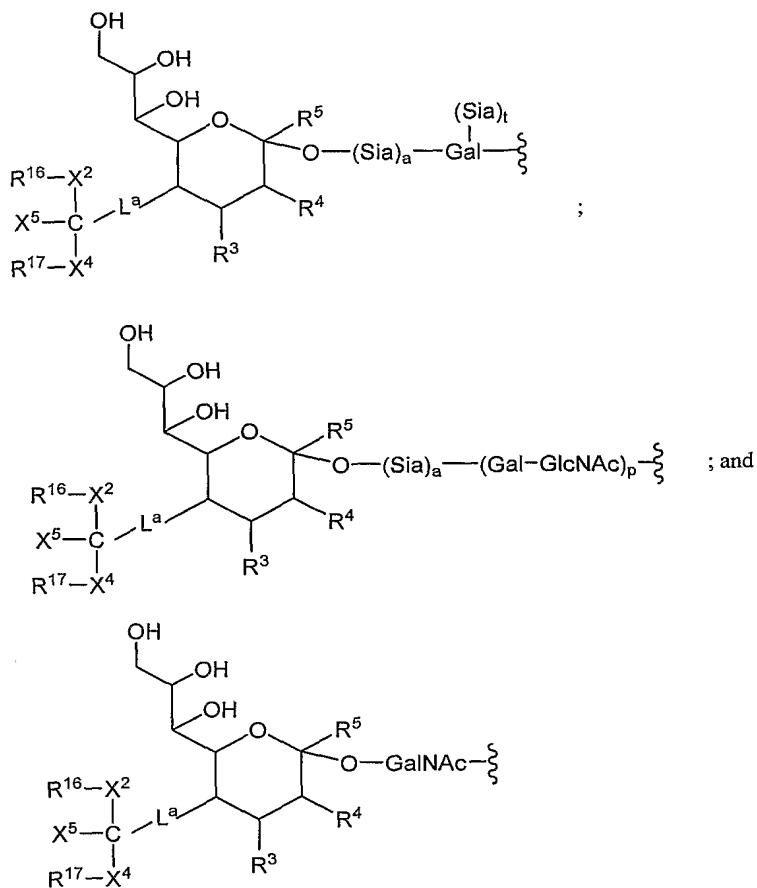
[0155] In another exemplary embodiment, the lipid or peptide comprises a glycosyl moiety

5 selected from the formulae:



in which L^a is a bond or a linker as described herein; the index t represents 0 or 1; and the index a represents 0 or 1. Each of these groups can be included as components of the mono-, bi-, tri- and tetra-antennary saccharide structures set forth above.

10 **[0156]** In yet another embodiment, the lipid or peptide conjugates of the invention include a modified glycosyl residue that includes the substructure selected from:

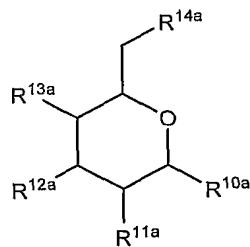


in which the index a and the linker L^a are as discussed above. The index p is an integer from 1 to 10. The indices t and a are independently selected from 0 or 1. Each of these groups can be included as components of the mono-, bi-, tri- and tetra-antennary saccharide structures set forth above.

5

[0157] In a further exemplary embodiment, the invention utilizes modified sugars in which the 6-hydroxyl position is converted to the corresponding amine moiety, which bears a linker-modifying group cassette such as those set forth above. Exemplary glycosyl groups that can be used as the core of these modified sugars include Gal, GalNAc, Glc, GlcNAc, Fuc, Xyl,

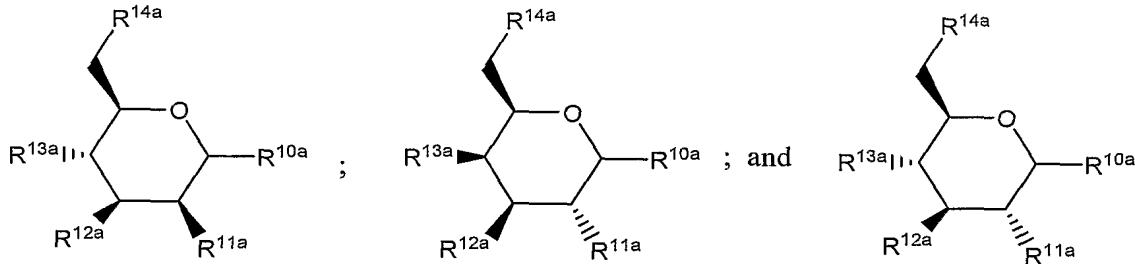
10 Man, and the like. A representative modified sugar according to this embodiment has the formula:



in which R^{11a} , R^{12a} , R^{13a} and R^{14a} are members independently selected from H, OH, $C(O)CH_3$, NH, and $NH\ C(O)CH_3$. R^{10a} is a link to another glycosyl residue (-O-glycosyl) or to an amino acid of the peptide (-NH-(peptide)). R^{14a} is OR^{10} or NHR^{10} . R^{10} is described above.

[0158] Selected conjugates according to this motif are based on mannose, galactose or

5 glucose, or on species having the stereochemistry of mannose, galactose or glucose. The general formulae of these conjugates are:



[0159] As discussed above, the invention provides saccharides bearing a modifying group,

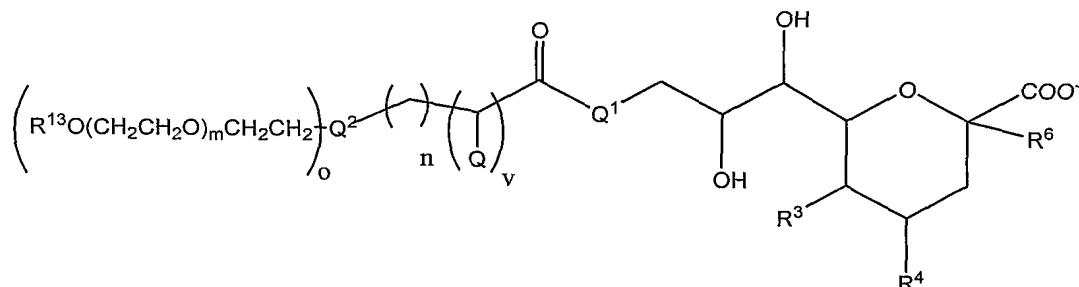
activated analogues of these species and conjugates formed between species such as peptides

10 and lipids and a modified saccharide of the invention.

[0160] Still further exemplary species of use in the invention are substrates for proteases.

Thus, it is within the scope of the invention to utilize of any one or more of the structures shown above, or analogues thereof, to form a conjugate of the invention. See, for example, WO 03/014371.

15 **[0161]** As discussed above, selected conjugates of the invention include one or more polymer moiety, such as PEG. Conjugates of the invention that include modifying groups that are PEG can be of the formula:



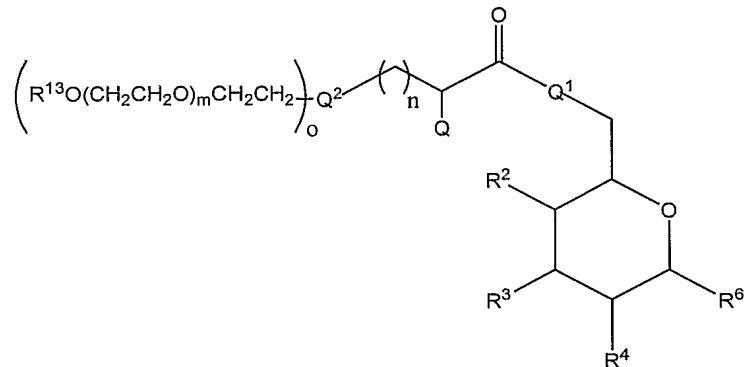
in which, the index "m" is an integer from 1 to 2500; the index n represents an integer from 0

20 to 40; and the symbol R^{13} represents H or substituted or unsubstituted alkyl. Q is H,

substituted or unsubstituted alkyl, or a side chain of an amino acid, or a linker to a polymer.

Q^1 is selected from O, S and species that include a nitrogen atom. Q^2 is O, S or NH. Alternatively, Q^2 is an amino acid or peptidyl residue. The index "o" is an integer from 1 to 4. When o is greater than 1, Q^2 is generally an amino acid or peptidyl residue as discussed herein and the PEG moiety is optionally a branched PEG moiety. The index v is either 0 or 5 1. In an exemplary conjugate according to this formula, the glycosyl residue is a sialic acid residue.

[0162] Yet another exemplary PEG conjugate has the formula:



in which the index m is an integer from 1 to 2500; the index n is an integer from 0 to 40; and 10 R^{13} is a member selected from H and substituted or unsubstituted alkyl. Q , Q^1 and Q^2 are as discussed above. Exemplary Q^2 species are linkers, e.g., urethane, amide, amino acid residue, peptidyl residue and the like.

[0163] The components of the conjugates of the invention are discussed in greater detail in the sections that follow.

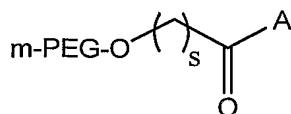
15 **Sugars**

[0164] Any sugar can be utilized as the sugar core of the conjugates of the invention. Exemplary sugar cores that are useful in forming the compositions of the invention include, but are not limited to, glucose, galactose, and mannose and N-acetyl analogues of these sugars. Also of use are fucose, xylose, ribose, arabinose, and sialic acid. Also encompassed 20 within the invention are species in which the sugar core is a disaccharide, an oligosaccharide or a polysaccharide. The sugar core can also be attached to an aglycone, such as a peptide, a lipid, or a sugar nucleotide (such as cytosine monophosphate (CMP), uracil diphosphate (UDP), and guanosine diphosphate (GDP)).

Modifying Groups and Activated Modifying Groups

[0165] Another glyco-conjugate component is the modifying group. Prior to the enzymatic reaction, these groups are covalently attached to a leaving group, thus forming an activated modifying group. In an exemplary embodiment, the activated modifying group has the

5 structure:



In which the index "s" represents an integer from 1 to 20; and A is any activating group that can be removed by one or more lipase, protease, acyltransferase, esterase or acylase in the process of transferring the acyl donor moiety to the saccharide. A is an activating group,

10 such as a leaving group. Exemplary leaving groups include allyl groups, $\text{CH}_2\text{CH}=\text{CH}_2$, O-aryl, active esters, S-alkyl, S-aryl and the like. Through the enzyme catalyzed reaction, the leaving group of the activated modifying group is displaced, and a covalent bond is formed between the sugar moiety and the modifying group, thus forming the glyco-conjugate.

Modifying Groups

15 [0166] The modifying groups of the invention can be any group, *e.g.*, water-soluble polymer, water-insoluble polymer, therapeutic moiety, etc., that can be conjugated to a sugar moiety through the use of the enzymes described herein.

Water-Soluble Polymers

[0167] Many water-soluble polymers are known to those of skill in the art and are useful in practicing the present invention. The term water-soluble polymer encompasses species such as saccharides (*e.g.*, dextran, amylose, hyalouronic acid, poly(sialic acid), heparans, heparins, etc.); poly (amino acids), *e.g.*, poly(aspartic acid) and poly(glutamic acid); nucleic acids; synthetic polymers (*e.g.*, poly(acrylic acid), poly(ethers), *e.g.*, poly(ethylene glycol); peptides, proteins, and the like. The present invention may be practiced with any water-soluble polymer with the sole limitation that the polymer must include a point at which the remainder of the conjugate can be attached.

25 [0168] Methods for activation of polymers can also be found in WO 94/17039, U.S. Pat. No. 5,324,844, WO 94/18247, WO 94/04193, U.S. Pat. No. 5,219,564, U.S. Pat. No. 5,122,614, WO 90/13540, U.S. Pat. No. 5,281,698, and more WO 93/15189, and for 30 conjugation between activated polymers and peptides, *e.g.* Coagulation Factor VIII (WO

94/15625), hemoglobin (WO 94/09027), oxygen carrying molecule (U.S. Pat. No. 4,412,989), ribonuclease and superoxide dismutase (Veronese *et al.*, *App. Biochem. Biotech.* **11**: 141-45 (1985)).

5 [0169] Preferred water-soluble polymers are those in which a substantial proportion of the polymer molecules in a sample of the polymer are of approximately the same molecular weight; such polymers are “homodisperse.”

10 [0170] The present invention is further illustrated by reference to a poly(ethylene glycol) conjugate. Several reviews and monographs on the functionalization and conjugation of PEG are available. *See, for example, Harris, Macromol. Chem. Phys.* **C25**: 325-373 (1985); Scouten, *Methods in Enzymology* **135**: 30-65 (1987); Wong *et al.*, *Enzyme Microb. Technol.* **14**: 866-874 (1992); Delgado *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems* **9**: 249-304 (1992); Zalipsky, *Bioconjugate Chem.* **6**: 150-165 (1995); and Bhadra, *et al.*, *Pharmazie*, **57**:5-29 (2002). Routes for preparing reactive PEG molecules and forming conjugates using the reactive molecules are known in the art. For example, U.S. Patent No. 15 5,672,662 discloses a water soluble and isolatable conjugate of an active ester of a polymer acid selected from linear or branched poly(alkylene oxides), poly(oxyethylated polyols), poly(olefinic alcohols), and poly(acrylomorpholine).

20 [0171] U.S. Patent No. 6,376,604 sets forth a method for preparing a water-soluble 1-benzotriazolylcarbonate ester of a water-soluble and non-peptidic polymer by reacting a terminal hydroxyl of the polymer with di(1-benzotriazoyl)carbonate in an organic solvent. The active ester is used to form conjugates with a biologically active agent such as a protein or peptide.

25 [0172] WO 99/45964 describes a conjugate comprising a biologically active agent and an activated water soluble polymer comprising a polymer backbone having at least one terminus linked to the polymer backbone through a stable linkage, wherein at least one terminus comprises a branching moiety having proximal reactive groups linked to the branching moiety, in which the biologically active agent is linked to at least one of the proximal reactive groups. Other branched poly(ethylene glycols) are described in WO 96/21469, U.S. Patent No. 5,932,462 describes a conjugate formed with a branched PEG molecule that includes a 30 branched terminus that includes reactive functional groups. The free reactive groups are available to react with a biologically active species, such as a protein or peptide, forming conjugates between the poly(ethylene glycol) and the biologically active species. U.S. Patent

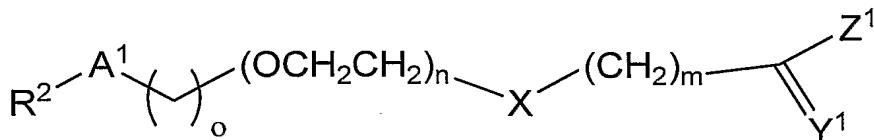
No. 5,446,090 describes a bifunctional PEG linker and its use in forming conjugates having a peptide at each of the PEG linker termini.

[0173] Conjugates that include degradable PEG linkages are described in WO 99/34833; and WO 99/14259, as well as in U.S. Patent No. 6,348,558. Such degradable linkages are

5 applicable in the present invention.

[0174] Although both reactive PEG derivatives and conjugates formed using the derivatives are known in the art, until the present invention, it was not recognized that a conjugate could be formed selectively between a specific site on a glycopeptide or glycolipid and PEG (or other polymer) through an intact glycosyl linking group.

10 **[0175]** In another exemplary embodiment, poly(ethylene glycol) molecules of use in the invention include, but are not limited to, those species set forth below.



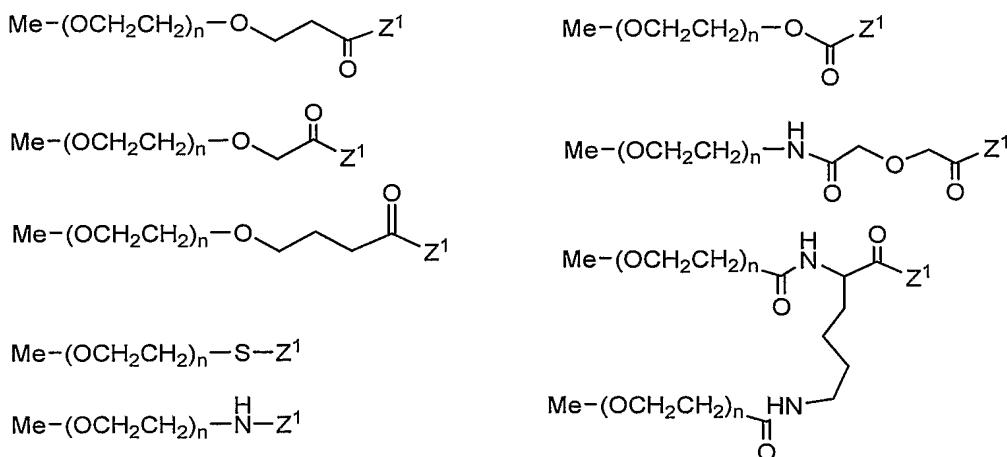
in which R^2 is H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl,

15 substituted or unsubstituted heteroalkyl, e.g., acetal, OHC- , $\text{H}_2\text{N}-(\text{CH}_2)_q-$, $\text{HS}-(\text{CH}_2)_q$, and $-(\text{CH}_2)_q\text{C}(\text{Y}^1)\text{Z}^2$; -sugar-nucleotide, or protein. The index "n" represents an integer from 1 to 2500. The indeces m, o, and q independently represent integers from 0 to 20. The symbols Z^1 and Z^2 independently represent OH, NH₂, halogen, S-R³, the alcohol portion of activated esters, $-(\text{CH}_2)_p\text{C}(\text{Y}^2)\text{V}$, $-(\text{CH}_2)_p\text{U}(\text{CH}_2)_s\text{C}(\text{Y}^2)\text{v}$, sugar-nucleotide, protein, and

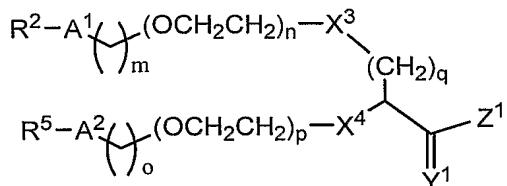
20 leaving groups, e.g., imidazole, p-nitrophenyl, HOBT, tetrazole, halide. The symbols X, Y¹, Y², A¹, and U independently represent the moieties O, S, N-R⁴. The symbol V represents OH, NH₂, halogen, S-R⁵, the alcohol component of activated esters, the amine component of activated amides, sugar-nucleotides, and proteins. The indeces p, q, s and v are members independently selected from the integers from 0 to 20. The symbols R³, R⁴ and R⁵

25 independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted heteroaryl.

[0176] In other exemplary embodiments, the poly(ethylene glycol) molecule is selected from the following:



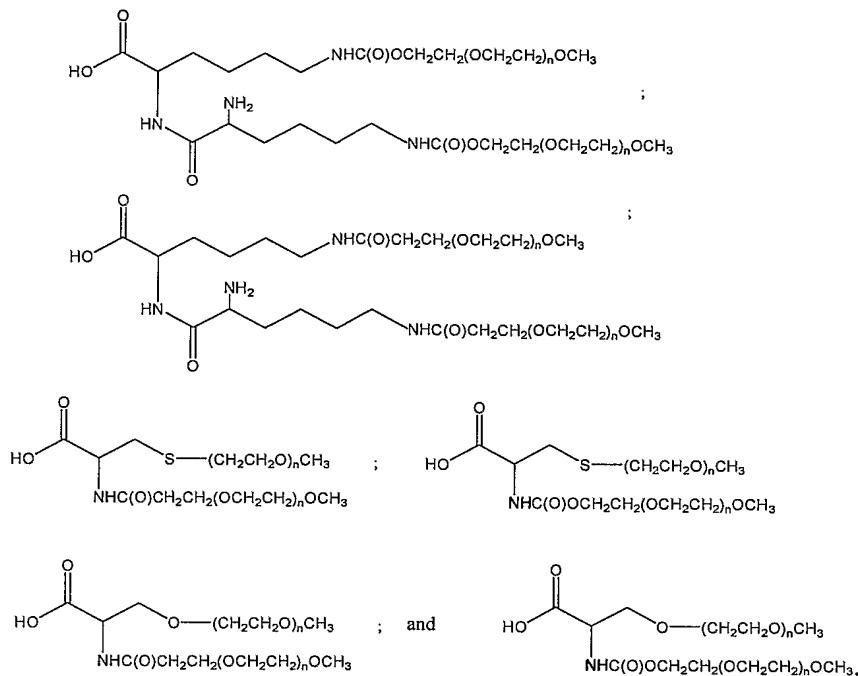
[0177] The poly(ethylene glycol) useful in forming the conjugate of the invention is either linear or branched. Branched poly(ethylene glycol) molecules suitable for use in the invention include, but are not limited to, those described by the following formula:



5

in which R^2 and R^5 are members independently selected from the groups defined for R^2 , above. A^1 and A^2 are members independently selected from the groups defined for A^1 , above. The indeces m , n , o , p and q are as described above. Z^1 and Y^1 are as described above. X^3 and X^4 are members independently selected from S , $SC(O)$, O , NH , $NHC(O)$ and $NHC(O)O$.

[0178] In other exemplary embodiments, the branched PEG is based upon a cysteine, serine di-lysine or tri-lysine core. Thus, further exemplary branched PEGs include:



[0179] In exemplary embodiments of the invention, the PEG is m-PEG (5 kD, 10 kD, or 20kD). An exemplary branched PEG species is a serine- or cysteine-(m-PEG)₂ in which the m-PEG is a 20 kD m-PEG.

5 **[0180]** As will be apparent to those of skill, the branched polymers of use in the invention include variations on the themes set forth above. For example the di-lysine-PEG conjugate shown above can include three polymeric subunits, the third bonded to the α -amine shown as unmodified in the structure above. Similarly, the use of a tri-lysine functionalized with three or four polymeric subunits is within the scope of the invention.

10 **[0181]** Those of skill in the art will appreciate that one or more of the mPEG arms of the branched polymer can be replaced by a PEG moiety with a different terminus, e.g., OH, COOH, NH₂, C₂-C₁₀-alkyl, etc. Moreover, the structures above are readily modified by inserting alkyl linkers (or removing carbon atoms) between the α -carbon atom and the functional group of the side chain. Thus, "homo" derivatives and higher homologues, as well 15 as lower homologues are within the scope of cores for branched PEGs of use in the present invention. Furthermore, one or more PEG moiety can be replaced by a modifying group other than a water-soluble polymer, e.g., therapeutic moiety, biomolecule, or water-insoluble polymer.

Water-Insoluble Polymers

[0182] In another embodiment, analogous to those discussed above, the modifying group is a water-insoluble polymer, rather than a water-soluble polymer. The glyco-conjugates of the invention may also include one or more water-insoluble polymers. This embodiment of the 5 invention is illustrated by the use of the conjugate as a vehicle with which to deliver a therapeutic peptide in a controlled manner. Polymeric drug delivery systems are known in the art. *See, for example, Dunn et al., Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991.* Those of skill in the art will appreciate that substantially any known drug delivery 10 system is applicable to the conjugates of the present invention.

[0183] Representative water-insoluble polymers include, but are not limited to, polyphosphazines, poly(vinyl alcohols), polyamides, polycarbonates, polyalkylenes, polyacrylamides, polyalkylene glycols, polyalkylene oxides, polyalkylene terephthalates, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyglycolides, 15 polysiloxanes, polyurethanes, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butyl methacrylate), poly(isobutyl methacrylate), poly(hexyl methacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly 20 (ethylene terephthalate), poly(vinyl acetate), polyvinyl chloride, polystyrene, polyvinyl pyrrolidone, pluronic and polyvinylphenol and copolymers thereof.

[0184] Synthetically modified natural polymers of use in the glyco-conjugates of the invention include, but are not limited to, alkyl celluloses, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, and nitrocelluloses. Particularly preferred members of the broad 25 classes of synthetically modified natural polymers include, but are not limited to, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxymethyl cellulose, cellulose triacetate, cellulose sulfate sodium salt, and polymers of acrylic and methacrylic esters and alginic acid.

30 [0185] These and the other polymers discussed herein can be readily obtained from commercial sources such as Sigma Chemical Co. (St. Louis, MO.), Polysciences (Warrenton,

PA.), Aldrich (Milwaukee, WI.), Fluka (Ronkonkoma, NY), and BioRad (Richmond, CA), or else synthesized from monomers obtained from these suppliers using standard techniques.

[0186] Representative biodegradable polymers of use in the conjugates of the invention include, but are not limited to, polylactides, polyglycolides and copolymers thereof, 5 poly(ethylene terephthalate), poly(butyric acid), poly(valeric acid), poly(lactide-co-caprolactone), poly(lactide-co-glycolide), polyanhydrides, polyorthoesters, blends and copolymers thereof. Of particular use are compositions that form gels, such as those including collagen, pluronic and the like.

[0187] The polymers of use in the invention include "hybrid" polymers that include water-10 insoluble materials having within at least a portion of their structure, a bioresorbable molecule. An example of such a polymer is one that includes a water-insoluble copolymer, which has a bioresorbable region, a hydrophilic region and a plurality of crosslinkable functional groups per polymer chain.

[0188] For purposes of the present invention, "water-insoluble materials" includes 15 materials that are substantially insoluble in water or water-containing environments. Thus, although certain regions or segments of the copolymer may be hydrophilic or even water-soluble, the polymer molecule, as a whole, is not substantially soluble in water.

[0189] For purposes of the present invention, the term "bioresorbable molecule" includes a region that is capable of being metabolized or broken down and resorbed and/or eliminated 20 through normal excretory routes by the body. Such metabolites or break down products are preferably substantially non-toxic to the body.

[0190] The bioresorbable region may be either hydrophobic or hydrophilic, so long as the copolymer composition as a whole is not rendered water-soluble. Thus, the bioresorbable region is selected based on the preference that the polymer, as a whole, remains water-25 insoluble. Accordingly, the relative properties, *i.e.*, the kinds of functional groups contained by, and the relative proportions of the bioresorbable region, and the hydrophilic region are selected to ensure that useful bioresorbable compositions remain water-insoluble.

[0191] Exemplary resorbable polymers include, for example, synthetically produced 30 resorbable block copolymers of poly(α -hydroxy-carboxylic acid)/poly(oxyalkylene, (*see*, Cohn *et al.*, U.S. Patent No. 4,826,945). These copolymers are not crosslinked and are water-soluble so that the body can excrete the degraded block copolymer compositions. *See*,

Younes *et al.*, *J Biomed. Mater. Res.* **21**: 1301-1316 (1987); and Cohn *et al.*, *J Biomed. Mater. Res.* **22**: 993-1009 (1988).

[0192] Presently preferred bioresorbable polymers include one or more components selected from poly(esters), poly(hydroxy acids), poly(lactones), poly(amides), poly(ester-amides), poly (amino acids), poly(anhydrides), poly(orthoesters), poly(carbonates), poly(phosphazines), poly(phosphoesters), poly(thioesters), polysaccharides and mixtures thereof. More preferably still, the biosresorbable polymer includes a poly(hydroxy) acid component. Of the poly(hydroxy) acids, polylactic acid, polyglycolic acid, polycaproic acid, polybutyric acid, polyvaleric acid and copolymers and mixtures thereof are preferred.

[0193] In addition to forming fragments that are absorbed *in vivo* ("bioresorbed"), preferred polymeric coatings for use in the methods of the invention can also form an excretable and/or metabolizable fragment.

[0194] Higher order copolymers can also be used in the present invention. For example, Casey *et al.*, U.S. Patent No. 4,438,253, which issued on March 20, 1984, discloses tri-block copolymers produced from the transesterification of poly(glycolic acid) and an hydroxyl-ended poly(alkylene glycol). Such compositions are disclosed for use as resorbable monofilament sutures. The flexibility of such compositions is controlled by the incorporation of an aromatic orthocarbonate, such as tetra-p-tolyl orthocarbonate into the copolymer structure.

[0195] Other polymers based on lactic and/or glycolic acids can also be utilized. For example, Spinu, U.S. Patent No. 5,202,413, which issued on April 13, 1993, discloses biodegradable multi-block copolymers having sequentially ordered blocks of polylactide and/or polyglycolide produced by ring-opening polymerization of lactide and/or glycolide onto either an oligomeric diol or a diamine residue followed by chain extension with a di-functional compound, such as, a diisocyanate, diacylchloride or dichlorosilane.

[0196] Bioresorbable regions of coatings useful in the present invention can be designed to be hydrolytically and/or enzymatically cleavable. For purposes of the present invention, "hydrolytically cleavable" refers to the susceptibility of the copolymer, especially the bioresorbable region, to hydrolysis in water or a water-containing environment. Similarly, "enzymatically cleavable" as used herein refers to the susceptibility of the copolymer, especially the bioresorbable region, to cleavage by endogenous or exogenous enzymes.

[0197] When placed within the body, the hydrophilic region can be processed into excretable and/or metabolizable fragments. Thus, the hydrophilic region can include, for example, polyethers, polyalkylene oxides, polyols, poly(vinyl pyrrolidine), poly(vinyl alcohol), poly(alkyl oxazolines), polysaccharides, carbohydrates, peptides, proteins and 5 copolymers and mixtures thereof. Furthermore, the hydrophilic region can also be, for example, a poly(alkylene) oxide. Such poly(alkylene) oxides can include, for example, poly(ethylene) oxide, poly(propylene) oxide and mixtures and copolymers thereof.

[0198] Polymers that are components of hydrogels are also useful in the present invention. Hydrogels are polymeric materials that are capable of absorbing relatively large quantities of 10 water. Examples of hydrogel forming compounds include, but are not limited to, polyacrylic acids, sodium carboxymethylcellulose, polyvinyl alcohol, polyvinyl pyrrolidine, gelatin, carrageenan and other polysaccharides, hydroxyethylenemethacrylic acid (HEMA), as well as derivatives thereof, and the like. Hydrogels can be produced that are stable, biodegradable and bioresorbable. Moreover, hydrogel compositions can include subunits that exhibit one or 15 more of these properties.

[0199] Bio-compatible hydrogel compositions whose integrity can be controlled through crosslinking are known and are presently preferred for use in the methods of the invention. For example, Hubbell *et al.*, U.S. Patent Nos. 5,410,016, which issued on April 25, 1995 and 5,529,914, which issued on June 25, 1996, disclose water-soluble systems, which are 20 crosslinked block copolymers having a water-soluble central block segment sandwiched between two hydrolytically labile extensions. Such copolymers are further end-capped with photopolymerizable acrylate functionalities. When crosslinked, these systems become hydrogels. The water soluble central block of such copolymers can include poly(ethylene glycol); whereas, the hydrolytically labile extensions can be a poly(α -hydroxy acid), such as 25 polyglycolic acid or polylactic acid. *See*, Sawhney *et al.*, *Macromolecules* **26**: 581-587 (1993).

[0200] In another preferred embodiment, the gel is a thermoreversible gel. Thermoreversible gels including components, such as pluronic, collagen, gelatin, hyalouronic acid, polysaccharides, polyurethane hydrogel, polyurethane-urea hydrogel and 30 combinations thereof are presently preferred.

[0201] In yet another exemplary embodiment, the conjugate of the invention includes a component of a liposome. Liposomes can be prepared according to methods known to those

skilled in the art, for example, as described in Eppstein *et al.*, U.S. Patent No. 4,522,811, which issued on June 11, 1985. For example, liposome formulations may be prepared by dissolving appropriate lipid(s) (such as stearoyl phosphatidyl ethanolamine, stearoyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on the surface of the container. An aqueous solution of the active compound or its pharmaceutically acceptable salt is then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

10 [0202] The above-recited microparticles and methods of preparing the microparticles are offered by way of example and they are not intended to define the scope of microparticles of use in the present invention. It will be apparent to those of skill in the art that an array of microparticles, fabricated by different methods, are of use in the present invention.

Methods

15 [0203] In addition to the compositions discussed above, the present invention provides methods for preparing glyco-conjugates. Moreover, the invention provides methods of preventing, curing or ameliorating a disease state by administering a conjugate of the invention to a subject at risk of developing the disease or a subject that has the disease.

20 [0204] Thus, the invention provides a method of forming a glyco-conjugate between a modifying group and a glycosyl-containing compound, *e.g.*, a glycopeptide, or a glycolipid. For clarity of illustration, the invention is illustrated with reference to a conjugate formed between a glycopeptide and an activated modifying group that includes a water-soluble polymer. Those of skill will appreciate that the invention equally encompasses methods of forming conjugates of glycolipids with water-soluble polymers, and forming conjugates 25 between glycopeptides and glycolipids and modifying groups other than water-soluble polymers.

[0205] In a representative embodiment, the method includes: (a) contacting a peptide comprising a glycosyl residue with;

30 (i) an acylating agent comprising an activated acyl moiety that is reactive with an N-, O- or S-containing residue on the peptide; and
(ii) an enzyme for which said acylating agent is a substrate, under conditions appropriate to acylate said glycosyl residue.

[0206] In exemplary embodiments, the conjugate is formed between a water-soluble polymer, a therapeutic moiety, targeting moiety or a biomolecule, and a glycosylated peptide. The polymer, therapeutic moiety or biomolecule is conjugated to the peptide via a glycosyl linking group, which is interposed between, and covalently linked to, both the peptide
5 (directly or through an intervening glycosyl linker) and the modifying group (e.g., water-soluble polymer). The method includes contacting the glycopeptide with an activated modifying group and an enzyme for which the activated modifying group is a substrate. The components of the reaction mixture are combined under conditions appropriate to acylate a selected glycosyl residue on the glycopeptide, thereby preparing the conjugate. In an
10 exemplary embodiment, the glycosyl residue is acylated by the acylating agent at a site that is a member selected from an OH, NH₂ and SH.

[0207] The acceptor peptide is typically synthesized *de novo*, or recombinantly expressed in a prokaryotic cell (e.g., bacterial cell, such as *E. coli*) or in a eukaryotic cell such as a mammalian, yeast, insect, fungal or plant cell. The peptide can be either a full-length protein
15 or a fragment. Moreover, the peptide can be a wild type or mutated peptide. In an exemplary embodiment, the peptide includes a mutation that adds one or more N- or O-linked glycosylation sites to the peptide sequence.

[0208] The method of the invention also provides for modification of incompletely glycosylated peptides that are produced recombinantly. Many recombinantly produced
20 glycoproteins are incompletely glycosylated, exposing carbohydrate residues that may have undesirable properties, e.g., immunogenicity, recognition by the RES. The incomplete glycosyl residue can be masked using a water-soluble polymer.

[0209] Those of skill will appreciate that the invention can be practiced using substantially any peptide or glycopeptide from any source. Exemplary peptides with which
25 the invention can be practiced are set forth in WO 03/031464, and the references set forth therein.

[0210] Peptides modified by the methods of the invention can be synthetic or wild-type peptides or they can be mutated peptides, produced by methods known in the art, such as site-directed mutagenesis. Glycosylation of peptides is typically either N-linked or O-linked. An
30 exemplary N-linkage is the attachment of the modified sugar to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for

enzymatic attachment of a carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one sugar (e.g., N-acetylgalactosamine, galactose, mannose, GlcNAc, glucose, fucose or xylose) to the hydroxy side chain of a hydroxyamino acid, preferably serine or threonine, although unusual or non-natural amino acids, e.g., 5-hydroxyproline or 5-hydroxylysine may also be used.

[0211] Moreover, in addition to peptides, the methods of the present invention can be practiced with other biological structures (e.g., glycolipids, lipids, sphingoids, ceramides, whole cells, and the like, containing a glycosylation site).

[0212] Addition of glycosylation sites to a peptide or other structure is conveniently accomplished by altering the amino acid sequence such that it contains one or more glycosylation sites. The addition may also be made by the incorporation of one or more species presenting an -OH group, preferably serine or threonine residues, within the sequence of the peptide (for O-linked glycosylation sites). The addition may be made by mutation or by full chemical synthesis of the peptide. The peptide amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the peptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) are preferably made using methods known in the art.

[0213] In an exemplary embodiment, the glycosylation site is added by shuffling polynucleotides. Polynucleotides encoding a candidate peptide can be modulated with DNA shuffling protocols. DNA shuffling is a process of recursive recombination and mutation, performed by random fragmentation of a pool of related genes, followed by reassembly of the fragments by a polymerase chain reaction-like process. *See, e.g., Stemmer, Proc. Natl. Acad. Sci. USA 91:10747-10751 (1994); Stemmer, Nature 370:389-391 (1994); and U.S. Patent Nos. 5,605,793, 5,837,458, 5,830,721 and 5,811,238.*

[0214] The present invention also provides means of adding (or removing) one or more selected glycosyl residues to a peptide, after which a modified sugar is conjugated to at least one of the selected glycosyl residues of the peptide. The present embodiment is useful, for example, when it is desired to conjugate the modified sugar to a selected glycosyl residue that is either not present on a peptide or is not present in a desired amount. Thus, prior to coupling a modified sugar to a peptide, the selected glycosyl residue is conjugated to the peptide by enzymatic or chemical coupling. In another embodiment, the glycosylation

pattern of a glycopeptide is altered prior to the conjugation of the modified sugar by the removal of a carbohydrate residue from the glycopeptide. *See, for example WO 98/31826.*

[0215] Addition or removal of any carbohydrate moieties present on the glycopeptide is accomplished either chemically or enzymatically. Chemical deglycosylation is preferably brought about by exposure of the polypeptide variant to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the peptide intact. Chemical deglycosylation is described by Hakimuddin *et al.*, *Arch. Biochem. Biophys.* **259**: 52 (1987) and by Edge *et al.*, *Anal. Biochem.* **118**: 131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptide variants can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al.*, *Meth. Enzymol.* **138**: 350 (1987).

[0216] Chemical addition of glycosyl moieties is carried out by any art-recognized method. Enzymatic addition of sugar moieties is preferably achieved using a modification of the methods set forth herein, substituting native glycosyl units for the modified sugars used in the invention. Other methods of adding sugar moieties are disclosed in U.S. Patent No. 5,876,980, 6,030,815, 5,728,554, and 5,922,577.

[0217] Exemplary attachment points for selected glycosyl residue include, but are not limited to: (a) consensus sites for N-linked glycosylation, and sites for O-linked glycosylation; (b) terminal glycosyl moieties that are acceptors for a glycosyltransferase; (c) arginine, asparagine and histidine; (d) free carboxyl groups; (e) free sulfhydryl groups such as those of cysteine; (f) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (g) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (h) the amide group of glutamine. Exemplary methods of use in the present invention are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston, CRC CRIT. REV. BIOCHEM., pp. 259-306 (1981).

[0218] In one embodiment, the invention provides a method for linking two or more peptides through a linking group. The linking group is of any useful structure and may be selected from straight- and branched-chain structures. Preferably, each terminus of the linker, which is attached to a peptide, includes a modified sugar.

[0219] In an exemplary method of the invention, two peptides are linked together via a linker moiety that includes a polymeric (e.g., PEG linker). The focus on a PEG linker that

includes two glycosyl groups is for purposes of clarity and should not be interpreted as limiting the identity of linker arms of use in this embodiment of the invention.

[0220] Exemplary peptides with which the present invention can be practiced, methods of adding or removing glycosylation sites, and adding or removing glycosyl structures or substructures are described in detail in WO03/031464 and related U.S. and PCT applications.

[0221] In addition to the compositions discussed above, the present invention provides methods for preparing peptide-conjugates comprising a lipid-based linker and a modifying group. Moreover, the invention provides methods of preventing, curing or ameliorating a disease state by administering a conjugate of the invention to a subject at risk of developing the disease or to a subject who has the disease.

[0222] Thus, the invention provides a method of forming a peptide-conjugate between a modifying group and a lipid-containing compound, *e.g.*, a lipopeptide. For clarity of illustration, the invention is illustrated with reference to a conjugate formed between a peptide and an activated modifying group comprising a lipid and a water-soluble polymer. Those of skill will appreciate that the invention equally encompasses methods of forming conjugates between peptides and modifying groups other than water-soluble polymers.

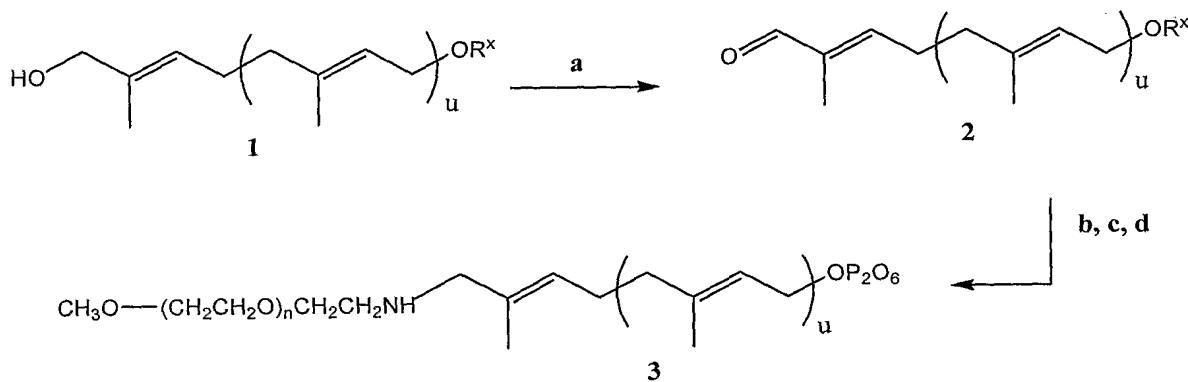
[0223] In exemplary embodiments, the conjugate is formed between a water-soluble polymer, a therapeutic moiety, targeting moiety or a biomolecule, and a peptide. The polymer, therapeutic moiety or biomolecule is conjugated to the peptide via a lipid linking group, which is interposed between, and covalently linked to, both the peptide and the modifying group (*e.g.*, water-soluble polymer). The method includes contacting the peptide with an activated modifying group comprising a lipid linker and a water-soluble polymer, and an enzyme for which the activated modifying group is a substrate. The components of the reaction mixture are combined under conditions appropriate to link the amino acid residue on the peptide, to the activated lipid linker comprising the modifying group thereby preparing the conjugate.

[0224] In one embodiment, the lipid linker is a fatty acid derivative comprising repeating methylene units. In this embodiment, the fatty acid may be linked to the peptide by a thioester bond with cysteine (*i.e.* thio-palmitoylation) or in amide linkage to an N-terminal glycine (*N*-acylation; Knoll *et al.* Methods in Enzymol. 250:405 (1995)) or an ϵ -amine of an internal lysine (Hackett M. *et al.* Science 266:433-435 (1994)).

[0225] In another embodiment, the lipid linker is a fatty acid comprising repeating isoprene units. In this embodiment, the peptide conjugate may comprise one or more modified lipids linked through one or more thioester linkages with cysteine residues of the peptide. In one aspect, modified lipids for use in the invention may be prepared according to one or more of 5 the methods outlined in Scheme 1-3 below.

[0226] Scheme 1 sets forth an exemplary route to PEGylated isoprenyl compounds of use in the present invention. Starting compound 1 is produced by protecting a commercially available alcohol (e.g., farnesol, geraniol). The selection of an appropriate protecting agent is within the ability of those of skill in the art.

10 **Scheme 1**

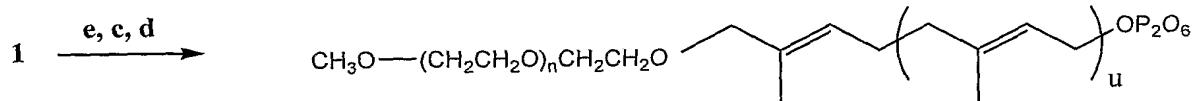


[0227] The protected alcohol is then selectively oxidized to compound 1 using an art-recognized method. See, e.g., Bukhtiyarov et al., *J. Biol. Chem.*, **270**: 19035-19040 (1995). For example, the alcohol can be formed by the action of t-butyl hydroperoxide and H₂SeO₃.

[0228] In step **a**, the unprotected hydroxyl moiety is selectively oxidized to the corresponding aldehyde. Exemplary oxidation conditions include catalytic oxidation using a supported platinum group metal ion, e.g., Ru-Al-Mg Hydrotalcite, Ru-Al-Co hydrotalcite, Pd(II) hydrotalcite, Pd Cluster Complex/TiO₂ and the like. The resulting carbonyl compound, e.g., aldehyde, is reductively aminated with m-PEG-amine (**b**), and the 15 protecting group is removed (**c**). The exposed hydroxyl moiety is converted to the corresponding diphosphate (**d**). See, Holloway et al., *Biochem. J.*, **104**: 57-70 (1967). Exemplary phosphorylation conditions for converting the hydroxyl to the diphosphate are bis-(triethylammonium)hydrogen phosphate in the presence of a large excess of CCl₃CN in 20 acetonitrile (Bukhtiyarov et al., *supra*).

[0229] Scheme 2 sets forth a route to compounds of use in a method of the invention in which the m-PEG moiety is tethered to the isoprenyl moiety through an ether linkage. Thus, alcohol 1 is reacted with an activated m-PEG species, e.g., a halo or sulfonate derivative under conditions appropriate to form the ether (e). The protecting group is removed (c) and 5 the resulting alcohol is phosphorylated as discussed above.

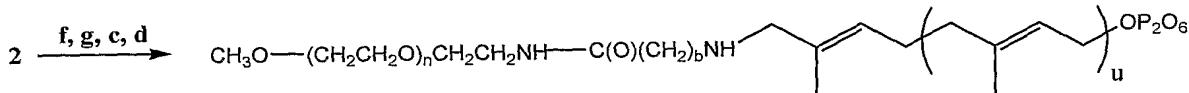
Scheme 2



[0230] Alternatively, a reactive starting material can be assembled using other recognized methods. See, for example, Mehta et al., *The Chemistry of Dienes and Polyenes*, Wiley 10 Interscience, NY, 1997.

[0231] In another embodiment, a linker is interposed between the m-PEG moiety and the isoprenyl moiety. An exemplary linker is based upon a amino carboxylic acid. Thus, according to Scheme 3, aldehyde 2 is reductively aminated with an amino carboxylic acid (f). The acid is activated, e.g., active ester, acid halide, and coupled with m-PEG amine, forming 15 the corresponding amide (g). The protecting group on the hydroxyl of the amide is removed (c) and the hydroxyl moiety is phosphorylated.

Scheme 3



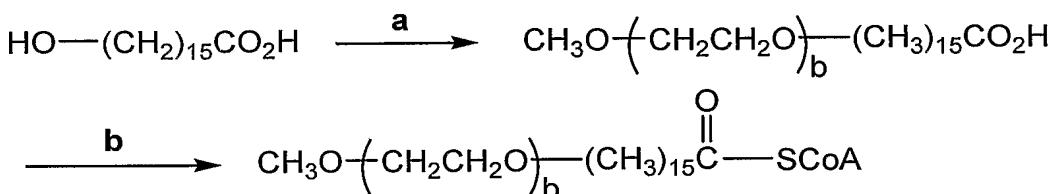
[0232] In another embodiment, the lipid linker is a fatty acid comprising repeating 20 methylene units. In this embodiment, the peptide conjugate may comprise one or more modified lipids linked through one or more amide linkages, e.g., on the α -amino group of an N-terminal glycine, or the ϵ -amino group of an internal lysine. In a related embodiment, the peptide conjugate may comprise one or more modified lipids comprised of repeating methylene units, and these lipids may be linked through one or more thioester linkages with 25 cysteine residues of the peptide. In a further related embodiment, the peptide conjugate may comprise one or more modified lipids comprised of repeating methylene units, and the modified lipids may be independently linked through both amide and thioester linkages on the same peptide. In one aspect, modified lipids for use in these embodiments of the invention may be prepared according to one or more of the methods outlined in Scheme 4.

[0233] Derivatives of palmitic acid can be activated for use with a transferase by converting the carboxylic group to a thioester. In an exemplary embodiment, set forth in scheme 4, the thioester is a CoA thioester. In Scheme 4, 16-OH palmitic acid is reacted with an activated poly(ethylene glycol) species under conditions appropriate for the formation of the corresponding ether. The carboxylic acid of the resulting PEG-palmitic acid ether is activated by conversion to an activated ester (e.g., NHS), an anhydride or the like. The activated species is converted to the corresponding Coenzyme A thioester by combining the activated species and Coenzyme A under conditions appropriate for the coupling to occur. The formation of CoA thioesters by this route and other analogous routes is known in the art.

5 See, for example, Kutner et al., *Proc. Natl. Acad. Sci. U.S.A.* **83**: 6781-4 (1986).

10

Scheme 4



a. $\text{CH}_3\text{O}-\left(\text{CH}_2\text{CH}_2\text{O}\right)_b-\text{CH}_2\text{CH}_2\text{Cl}$; NaH

b. (1) N-hydroxysuccinimide ; DCC : (2) Coenzyme A

[0234] The acceptor peptide is typically synthesized *de novo*, or recombinantly expressed in a prokaryotic cell (e.g., bacterial cell, such as *E. coli*) or in a eukaryotic cell such as a mammalian, yeast, insect, fungal or plant cell. The peptide can be either a full-length protein or a fragment. Moreover, the peptide can be a wild type or mutated peptide.

15

[0235] Exemplary peptides that can be modified using the methods of the invention are set forth in Table 1.

Table 1

<u>Hormones and Growth Factors</u>	<u>Receptors and Chimeric Receptors</u>
• G-CSF	• CD4
• GM-CSF	• Tumor Necrosis Factor (TNF) receptor
• M-CSF	• Alpha-CD20
• TPO	• MAb-CD20
• EPO	• MAb-alpha-CD3
• EPO variants	• MAb-TNF receptor
• alpha-TNF	• MAb-CD4

<ul style="list-style-type: none"> • Leptin • <u>Enzymes and Inhibitors</u> <ul style="list-style-type: none"> • t-PA • t-PA variants • Urokinase • Factors VII, VIII, IX, X • Dnase • Glucocerebrosidase • Hirudin • α1 antitrypsin • Antithrombin III • <u>Cytokines and Chimeric Cytokines</u> <ul style="list-style-type: none"> • Interleukin-1 (IL-1), 1B, 2, 3, 4 • Interferon-alpha (IFN-alpha) • IFN-alpha-2b • IFN-beta • IFN-gamma • Chimeric diphtheria toxin-IL-2 	<ul style="list-style-type: none"> • PSGL-1 • MAb-PSGL-1 • Complement • GlyCAM or its chimera • N-CAM or its chimera <p>Monoclonal Antibodies</p> <p>(Immunoglobulins)</p> <ul style="list-style-type: none"> • MAb-anti-RSV • MAb-anti-IL-2 receptor • MAb-anti-CEA • MAb-anti-platelet IIb/IIIa receptor • MAb-anti-EGF • MAb-anti-Her-2 receptor <p>Cells</p> <ul style="list-style-type: none"> • Red blood cells • White blood cells (<i>e.g.</i>, T cells, B cells, dendritic cells, macrophages, NK cells, neutrophils, monocytes and the like) • Stem cells
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[0236] Other exemplary peptides that are modified by the methods of the invention include members of the immunoglobulin family (*e.g.*, antibodies, MHC molecules, T cell receptors, and the like), intercellular receptors (*e.g.*, integrins, receptors for hormones or growth factors and the like) lectins, and cytokines (*e.g.*, interleukins). Additional examples include

5 tissue-type plasminogen activator (t-PA), renin, clotting factors such as factors V-XII, bombesin, thrombin, hematopoietic growth factor, colony stimulating factors, viral antigens, complement proteins, α 1-antitrypsin, erythropoietin, P-selectin glycopeptide ligand-1 (PSGL-1), granulocyte-macrophage colony stimulating factor, anti-thrombin III, interleukins, interferons, proteins A and C, fibrinogen, herceptin, leptin, glycosidases, HS-glycoprotein, 10 serum proteins (*e.g.*, α -acid glycoprotein, fetuin, α -fetal protein), β 2-glycoprotein, NeuroTropin III (NT III), Bone Morphogenic Peptide (BMP), BMP-II, Fibroblast Growth Factor (FGF), FGF-20, glutaminase-interacting protein (GIP), among many others. This list of polypeptides is exemplary, not exclusive. The methods are also useful for modifying fusion and chimeric proteins, including, but not limited to, chimeric proteins that include a 15 moiety derived from an immunoglobulin, such as IgG, or a fragment of an immunoglobin, *e.g.*, FAb (Fc domain). The exemplary peptides provided herein are intended to provide a selection of the peptides with which the present invention can be practiced; as such, they are

non-limiting. Those of skill will appreciate that the invention can be practiced using substantially any peptide from any source.

5 [0237] Peptides modified by the methods of the invention can be synthetic or wild-type peptides or they can be mutated peptides, produced by methods known in the art, such as site-directed mutagenesis.

Enzyme Classes

10 [0238] Aspects of the present invention make use of enzymes that form a bond between an activated acyl moiety and a heteroatom found on a sugar nucleus. The enzymes useful in practicing the present invention include, but are not limited to, wild-type and mutant proteases, lipases, esterases, acylases, acyltransferases, glycosyltransferases, sulfotransferases, glycosidases, and the like. An exemplary mutant is one in which one or more amino acid residues in the active site are altered to provide an enzyme with synthetic activity that is improved relative to the activity in the corresponding wild-type enzyme. In an exemplary 15 embodiment, the enzyme is a member selected from a lipase, a protease, an esterase, an acylase and an acyltransferase. In another exemplary embodiment, the enzyme has an amino acid sequence that is a wild-type sequence for said enzyme. In another exemplary embodiment, the enzyme is a mutated enzyme which has a mutated amino acid sequence. In another exemplary embodiment, the mutated enzyme has an acylation activity that is enhanced relative to a corresponding wild-type enzyme. In another exemplary embodiment, 20 the mutated amino acid sequence comprises a mutation wherein an amino acid residue implicated in hydrolysis of a member selected from an amide and an ester, is replaced by an amino acid residue that is not implicated in the hydrolysis.

Acyl Transfer

25 [0239] The discovery that some enzymes are catalytically active in organic solvents has greatly expanded their use as biocatalysts. In this medium these enzymes show a new catalytic behavior. For example lipases catalyse esterification and transesterification reactions in organic media. These properties enable the production of compounds which are difficult to obtain using chemical methods.

30 Proteases

[0240] A protease is employed in some embodiments of the invention. Proteases are known in the art to catalyze the attachment of amino acids to sugars through esterification. (Davis, (WO 03/014371, published Feb. 20, 2003). In this publication, a vinyl ester amino

acid group was reacted with a carbohydrate acyl acceptor in the presence of the serine protease subtilisin derived from *Bacillus lenthus*. Wild-type proteases can be additionally be isolated from *Bacillus amyloliquefaciens*. Mutant proteases can be made according to the teachings of, for example, PCT Publication Nos. WO 95/10615 and WO 91/06637, which are hereby incorporated by reference. Other proteases of use in this invention include serine proteases (such as chymotrypsin, plasmin, and thrombin), cysteine proteases (such as cathepsin B and papain), and aspartic endopeptidases (such as pepsin A, chymosin, cathepsin D).

[0241] In an exemplary embodiment, utilizing a protease, the link between the sugar moiety and the modifying group is an amino acid that is derivatized with the modifying group. The sugar and amino acid are linked through an amide moiety formed by the protease.

Lipases

[0242] A lipase is used in some embodiments of the invention. The use of lipases in the acylation of saccharides has been previously reported. For example, regioselective acylations of alkyl β -D-xylopyranosides using lipase PS in organic solvents was reported by Lopez. (Lopez *et al.*, *J. Org. Chem.*, **59**, 7027-7032 (1994)). Another group also utilized lipase PS in order to catalyze the transfer of acetyl groups onto sialic acids in vinyl acetate. (Lo *et al.*, *Bioorg. Med. Chem. Lett.*, **9**, 709-712 (1999)). Regioselective disaccharide acylation in *tert*-butyl alcohol catalyzed by *Candida antarctica* lipase has also been reported. (Woudenberg van-Oosterom *et al.*, *Biotechnol. Bioeng.*, **49**, 328-333 (1996)). Immobilized versions of the *Candida antarctica* lipase have also been used to acylate hydroxypropyl cellulose in *tert*-butanol. (Sereti *et al.*, *Biotechnol Bioeng.*, **72**(4), 495-500 (2001)). Other lipases of use in this invention include lipoprotein lipase, triacylglycerol lipase, diglyceride lipase, and postheparin lipase.

Esterases

[0243] Esterases can also be used in some embodiments of the invention. Acetylation of cellobiose and cellulose was shown to be catalyzed in aqueous medium in the presence of isopropenyl acetate by an intracellular carboxylesterase from *Arthrobacter viscosus*. (Cui *et al.*, *Enzyme Microb. Technol.*, **24**, 200-208 (1999)). Another group acetylated the amino groups of chitobiose and chitotetraose in an aqueous solution of 3M sodium acetate using a chitin deacetylase from *Colletotrichum lindemuthianum* (Tokuyasu *et al.*, *Carbohydr. Res.*, **322**, 26-31 (1999)). A third group utilized acetylxyran esterase (AcXE) from *Schizophyllum commune* to catalyze acetyl group transfer to methyl β -D-xylopyranoside, methyl β -D-

cellobioside, methyl β -D-glucopyranoside, cellotetraose, 2-deoxy-D-glucose, D-mannose, β -1,4-mannobiose, β -1,4-mannopentaose, β -1,4-mannohexaose, β -1,4-xylobiose, and β -1,4-xylopentaose. (Biely *et al.*, *Biochimica et Biophysica Acta*, **1623**, 62-71 (2003)). Acetylation of secondary alcohols was also achieved by transesterification from vinyl acetate by a 5 feruloyl esterase from *Humicola insolens*. (Hatzakis *et al.*, *J. Mol. Catal., B Enzym.* **21**, 309-311 (2003)). Other esterases of use in this invention include choline esterase, sterol esterase, hydroxycinnamoyl esterase, acetylsalicylic acid esterase, and polyneuridine esterase.

Acylases

[0244] Acylases can also be used in some embodiments of the invention. Exemplary acylases of use in this invention include aminoacylase I, L-amino-acid acylase, penicillin acylase, acetyl-CoA acylase, acyl-lysine deacylase, aculeacin A acylase, succinyl-CoA acylase, and acetyl-aspartic deaminase.

Acetyltransferases

[0245] In another embodiment of the invention, acyl transfer is accomplished by an acetyltransferase. The use of acetyltransferases in the acylation of saccharides has been previously reported. O-acetylation at the 9 position of sialic acid has been shown to occur from the product of several genes in the COS cell system (Shi *et al.*, *Glycobiology*, **8**(2), 199-205 (1998)). Maltose O-acetyltransferase (MAT) from *Escherichia coli* is known to catalyze acetyl group transfer to the C6 positions of glucose and maltose. (Leggio *et al.*, *Biochemistry*, **42**, 5225-5235 (2003)). This same group also utilized galactoside acetyltransferase (GAT) to catalyze acetyl group transfer to galactosyl units. Other acetyltransferases of use in this invention include spermidine acetyltransferase, diamine N-acetyltransferase, and sialate O-acetyltransferase.

Sugar Transfer

[0246] In addition to the enzymes discussed above in the context of forming the acyl-linked conjugate, the glycosylation pattern of the conjugate and the starting substrates (*e.g.*, peptides, lipids) can be elaborated, trimmed back or otherwise modified by methods utilizing other enzymes. For example, in one embodiment, the glycosyl acceptor for the acyl moiety is conjugated to the peptide (or aglycone) or to a glycosyl residue on the peptide (or aglycone) 25 using an enzymatically-mediated sugar transfer reaction. The methods of remodeling peptides and lipids using enzymes that transfer a sugar donor to an acceptor are discussed in great detail in DeFrees, WO 03/031464 A2, published April 17, 2003. A brief summary of 30 selected enzymes of use in the present method is set forth below.

Glycosyltransferases

[0247] Glycosyltransferases catalyze the addition of activated sugars (donor NDP-sugars), in a step-wise fashion, to a protein, glycopeptide, lipid or glycolipid or to the non-reducing end of a growing oligosaccharide. N-linked glycopeptides are synthesized via a transferase 5 and a lipid-linked oligosaccharide donor Dol-PP-NAG₂Glc₃Man₉ in an en block transfer followed by trimming of the core. In this case the nature of the "core" saccharide is somewhat different from subsequent attachments. A very large number of glycosyltransferases are known in the art.

[0248] The glycosyltransferase to be used in the present invention may be any as long as it 10 can utilize the modified sugar as a sugar donor. Examples of such enzymes include Leloir pathway glycosyltransferase, such as galactosyltransferase, N-acetylglucosaminyltransferase, N-acetylgalactosaminyltransferase, fucosyltransferase, sialyltransferase, mannosyltransferase, xylosyltransferase, glucurononyltransferase and the like.

[0249] For enzymatic saccharide syntheses that involve glycosyltransferase reactions, 15 glycosyltransferase can be cloned, or isolated from any source. Many cloned glycosyltransferases are known, as are their polynucleotide sequences. *See, e.g., "The WWW Guide To Cloned Glycosyltransferases,"* Taniguchi et al., 2002, *Handbook of Glycosyltransferases and Related Genes*, Springer, Tokyo. Glycosyltransferase amino acid sequences and nucleotide sequences encoding glycosyltransferases from which the amino 20 acid sequences can be deduced are also found in various publicly available databases, including GenBank, Swiss-Prot, EMBL, and others.

[0250] Glycosyltransferases that can be employed in the methods of the invention include, but are not limited to, galactosyltransferases, fucosyltransferases, glucosyltransferases, N-acetylgalactosaminyltransferases, N-acetylglucosaminyltransferases, glucuronyltransferases, 25 sialyltransferases, mannosyltransferases, glucuronic acid transferases, galacturonic acid transferases, and oligoglycosyltransferases. Suitable glycosyltransferases include those obtained from eukaryotes, as well as from prokaryotes.

[0251] DNA encoding glycosyltransferases may be obtained by chemical synthesis, by screening reverse transcripts of mRNA from appropriate cells or cell line cultures, by 30 screening genomic libraries from appropriate cells, or by combinations of these procedures. Screening of mRNA or genomic DNA may be carried out with oligonucleotide probes generated from the glycosyltransferases gene sequence. Probes may be labeled with a detectable group such as a fluorescent group, a radioactive atom or a chemiluminescent group

in accordance with known procedures and used in conventional hybridization assays. In the alternative, glycosyltransferases gene sequences may be obtained by use of the polymerase chain reaction (PCR) procedure, with the PCR oligonucleotide primers being produced from the glycosyltransferases gene sequence. *See*, U.S. Pat. No. 4,683,195 to Mullis *et al.* and U.S.

5 Pat. No. 4,683,202 to Mullis.

[0252] The glycosyltransferase may be synthesized in host cells transformed with vectors containing DNA encoding the glycosyltransferases enzyme. Vectors are used either to amplify DNA encoding the glycosyltransferases enzyme and/or to express DNA which encodes the glycosyltransferases enzyme. An expression vector is a replicable DNA 10 construct in which a DNA sequence encoding the glycosyltransferases enzyme is operably linked to suitable control sequences capable of effecting the expression of the glycosyltransferases enzyme in a suitable host. The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control 15 transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants.

20 [0253] In an exemplary embodiment, the invention utilizes a prokaryotic enzyme. Such glycosyltransferases include enzymes involved in synthesis of lipooligosaccharides (LOS), which are produced by many gram negative bacteria (Preston *et al.*, *Critical Reviews in Microbiology* 23(3): 139-180 (1996)). Such enzymes include, but are not limited to, the proteins of the *rfa* operons of species such as *E. coli* and *Salmonella typhimurium*, which

25 include a β 1,6 galactosyltransferase and a β 1,3 galactosyltransferase (*see, e.g.*, EMBL Accession Nos. M80599 and M86935 (*E. coli*); EMBL Accession No. S56361 (*S. typhimurium*)), a glucosyltransferase (Swiss-Prot Accession No. P25740 (*E. coli*), an β 1,2-glucosyltransferase (*rfaJ*)(Swiss-Prot Accession No. P27129 (*E. coli*) and Swiss-Prot Accession No. P19817 (*S. typhimurium*)), and an β 1,2-N-acetylglucosaminyltransferase

30 (*rfaK*)(EMBL Accession No. U00039 (*E. coli*)). Other glycosyltransferases for which amino acid sequences are known include those that are encoded by operons such as *rfaB*, which have been characterized in organisms such as *Klebsiella pneumoniae*, *E. coli*, *Salmonella*

typhimurium, *Salmonella enterica*, *Yersinia enterocolitica*, *Mycobacterium leprae*, and the *rh1* operon of *Pseudomonas aeruginosa*.

[0254] Also suitable for use in the present invention are glycosyltransferases that are involved in producing structures containing lacto-N-neotetraose, D-galactosyl- β -1,4-N-acetyl-D-glucosaminyl- β -1,3-D-galactosyl- β -1,4-D-glucose, and the P^k blood group trisaccharide sequence, D-galactosyl- α -1,4-D-galactosyl- β -1,4-D-glucose, which have been identified in the LOS of the mucosal pathogens *Neisseria gonorrhoeae* and *N. meningitidis* (Scholten *et al.*, *J. Med. Microbiol.* **41**: 236-243 (1994)). The genes from *N. meningitidis* and *N. gonorrhoeae* that encode the glycosyltransferases involved in the biosynthesis of these structures have been identified from *N. meningitidis* immunotypes L3 and L1 (Jennings *et al.*, *Mol. Microbiol.* **18**: 729-740 (1995)) and the *N. gonorrhoeae* mutant F62 (Gotshlich, *J. Exp. Med.* **180**: 2181-2190 (1994)). In *N. meningitidis*, a locus consisting of three genes, *lgtA*, *lgtB* and *lgtE*, encodes the glycosyltransferase enzymes required for addition of the last three of the sugars in the lacto-N-neotetraose chain (Wakarchuk *et al.*, *J. Biol. Chem.* **271**: 19166-73 (1996)). Recently the enzymatic activity of the *lgtB* and *lgtA* gene product was demonstrated, providing the first direct evidence for their proposed glycosyltransferase function (Wakarchuk *et al.*, *J. Biol. Chem.* **271**(45): 28271-276 (1996)). In *N. gonorrhoeae*, there are two additional genes, *lgtD* which adds β -D-GalNAc to the 3 position of the terminal galactose of the lacto-N-neotetraose structure and *lgtC* which adds a terminal α -D-Gal to the lactose element of a truncated LOS, thus creating the P^k blood group antigen structure (Gotshlich (1994), *supra*). In *N. meningitidis*, a separate immunotype L1 also expresses the P^k blood group antigen and has been shown to carry an *lgtC* gene (Jennings *et al.*, (1995), *supra*). *Neisseria* glycosyltransferases and associated genes are also described in USPN 5,545,553 (Gotschlich). Genes for α 1,2-fucosyltransferase and α 1,3-fucosyltransferase from *Helicobacter pylori* has also been characterized (Martin *et al.*, *J. Biol. Chem.* **272**: 21349-21356 (1997)). Also of use in the present invention are the glycosyltransferases of *Campylobacter jejuni* (see, for example, http://afmb.cnrs-mrs.fr/~pedro/CAZY/gtf_42.html).

Fucosyltransferases

[0255] In some embodiments, a glycosyltransferase used in the method of the invention is a

fucosyltransferase. Fucosyltransferases are known to those of skill in the art. Exemplary fucosyltransferases include enzymes, which transfer L-fucose from GDP-fucose to a hydroxy position of an acceptor sugar. Fucosyltransferases that transfer non-nucleotide sugars to an acceptor are also of use in the present invention.

[0256] In some embodiments, the acceptor sugar is, for example, the GlcNAc in a Gal β (1 \rightarrow 3,4)GlcNAc β - group in an oligosaccharide glycoside. Suitable fucosyltransferases for this reaction include the Gal β (1 \rightarrow 3,4)GlcNAc β 1- α (1 \rightarrow 3,4)fucosyltransferase (FTIII E.C. No. 2.4.1.65), which was first characterized from human milk (see, Palcic, *et al.*,

5 *Carbohydrate Res.* **190**: 1-11 (1989); Prieels, *et al.*, *J. Biol. Chem.* **256**: 10456-10463 (1981); and Nunez, *et al.*, *Can. J. Chem.* **59**: 2086-2095 (1981)) and the Gal β (1 \rightarrow 4)GlcNAc β - α fucosyltransferases (FTIV, FTV, FTVI) which are found in human serum. FTVII (E.C. No. 2.4.1.65), a sialyl α (2 \rightarrow 3)Gal β ((1 \rightarrow 3)GlcNAc β fucosyltransferase, has also been characterized. A recombinant form of the Gal β (1 \rightarrow 3,4) GlcNAc β -

10 α (1 \rightarrow 3,4)fucosyltransferase has also been characterized (see, Dumas, *et al.*, *Bioorg. Med. Letters* **1**: 425-428 (1991) and Kukowska-Latallo, *et al.*, *Genes and Development* **4**: 1288-1303 (1990)). Other exemplary fucosyltransferases include, for example, α 1,2 fucosyltransferase (E.C. No. 2.4.1.69). Enzymatic fucosylation can be carried out by the methods described in Mollicone, *et al.*, *Eur. J. Biochem.* **191**: 169-176 (1990) or U.S. Patent

15 No. 5,374,655. Cells that are used to produce a fucosyltransferase will also include an enzymatic system for synthesizing GDP-fucose.

Galactosyltransferases

[0257] In another group of embodiments, the glycosyltransferase is a galactosyltransferase. Exemplary galactosyltransferases include α (1,3) galactosyltransferases (E.C. No. 2.4.1.151,

20 *see, e.g.*, Dabkowski *et al.*, *Transplant Proc.* **25**:2921 (1993) and Yamamoto *et al.* *Nature* **345**: 229-233 (1990), bovine (GenBank j04989, Joziasse *et al.*, *J. Biol. Chem.* **264**: 14290-14297 (1989)), murine (GenBank m26925; Larsen *et al.*, *Proc. Nat'l. Acad. Sci. USA* **86**: 8227-8231 (1989)), porcine (GenBank L36152; Strahan *et al.*, *Immunogenetics* **41**: 101-105 (1995)). Another suitable α 1,3 galactosyltransferase is that which is involved in synthesis of

25 the blood group B antigen (EC 2.4.1.37, Yamamoto *et al.*, *J. Biol. Chem.* **265**: 1146-1151 (1990) (human)). Yet a further exemplary galactosyltransferase is core Gal-T1.

[0258] Also suitable for use in the methods of the invention are β (1,4) galactosyltransferases, which include, for example, EC 2.4.1.90 (LacNAc synthetase) and EC 2.4.1.22 (lactose synthetase) (bovine (D'Agostaro *et al.*, *Eur. J. Biochem.* **183**: 211-217 (1989)), human (Masri *et al.*, *Biochem. Biophys. Res. Commun.* **157**: 657-663 (1988)), murine (Nakazawa *et al.*, *J. Biochem.* **104**: 165-168 (1988)), as well as E.C. 2.4.1.38 and the ceramide galactosyltransferase (EC 2.4.1.45, Stahl *et al.*, *J. Neurosci. Res.* **38**: 234-242

(1994)). Other suitable galactosyltransferases include, for example, α 1,2 galactosyltransferases (from *e.g.*, *Schizosaccharomyces pombe*, Chapell *et al.*, *Mol. Biol. Cell* 5: 519-528 (1994)).

Sialyltransferases

5 [0259] Sialyltransferases are another type of glycosyltransferase that is useful in the recombinant cells and reaction mixtures of the invention. Cells that produce recombinant sialyltransferases will also produce CMP-sialic acid, which is a sialic acid donor for sialyltransferases. Examples of sialyltransferases that are suitable for use in the present invention include ST3Gal III (*e.g.*, a rat or human ST3Gal III), ST3Gal IV, ST3Gal I, ST6Gal 10 I, ST3Gal V, ST6Gal II, ST6GalNAc I, ST6GalNAc II, and ST6GalNAc III (the sialyltransferase nomenclature used herein is as described in Tsuji *et al.*, *Glycobiology* 6: v-xiv (1996)). An exemplary α (2,3)sialyltransferase referred to as α (2,3)sialyltransferase (EC 2.4.99.6) transfers sialic acid to the non-reducing terminal Gal of a Gal β 1 \rightarrow 3Glc disaccharide or glycoside. *See*, Van den Eijnden *et al.*, *J. Biol. Chem.* 256: 3159 (1981), Weinstein *et al.*, 15 *J. Biol. Chem.* 257: 13845 (1982) and Wen *et al.*, *J. Biol. Chem.* 267: 21011 (1992). Another exemplary α 2,3-sialyltransferase (EC 2.4.99.4) transfers sialic acid to the non-reducing terminal Gal of the disaccharide or glycoside. *see*, Rearick *et al.*, *J. Biol. Chem.* 254: 4444 (1979) and Gillespie *et al.*, *J. Biol. Chem.* 267: 21004 (1992). Further exemplary enzymes include Gal- β -1,4-GlcNAc α -2,6 sialyltransferase (*See*, Kurosawa *et al.* *Eur. J. Biochem.* 20 219: 375-381 (1994)).

[0260] Preferably, for glycosylation of carbohydrates of glycopeptides the sialyltransferase will be able to transfer sialic acid to the sequence Gal β 1,4GlcNAc-, the most common penultimate sequence underlying the terminal sialic acid on fully sialylated carbohydrate structures (*see*, Table 1).

25 [0261] Eukaryotic sialyltransferases can also be used in the invention. Examples of suitable eukaryotic sialyltransferases for use in the present invention include ST3Gal III (*e.g.*, a rat or human ST3Gal III), ST3Gal IV, ST3Gal I, ST6Gal I, ST3Gal V, ST6Gal II, ST6GalNAc I, ST6GalNAc II, and ST6GalNAc III (the sialyltransferase nomenclature used herein is as described in Tsuji *et al.* (1996) *Glycobiology* 6: v-xiv). An exemplary 30 α (2,3)sialyltransferase referred to as α (2,3)sialyltransferase (EC 2.4.99.6) transfers sialic acid to the non-reducing terminal Gal of a Gal β 1 \rightarrow 3Glc disaccharide or glycoside. *See*, Van den Eijnden *et al.*, *J. Biol. Chem.*, 256:3159 (1981), Weinstein *et al.*, *J. Biol. Chem.*, 257:13845

(1982) and Wen *et al.*, *J. Biol. Chem.*, 267:21011 (1992). Another exemplary α 2,3-sialyltransferase (EC 2.4.99.4) transfers sialic acid to the non-reducing terminal Gal of the disaccharide or glycoside. See, Rearick *et al.*, *J. Biol. Chem.*, 254:4444 (1979) and Gillespie *et al.*, *J. Biol. Chem.*, 267:21004 (1992). Further exemplary enzymes include Gal- β -1,4-GlcNAc α -2,6 sialyltransferase (See, Kurosawa *et al.* *Eur. J. Biochem.* 219: 375-381 (1994)). Eukaryotic sialyltransferases generally comprise different functional domains, *e.g.*, a cytoplasmic domain, a signal-anchor domain, a stem region and a catalytic domain. In preferred embodiments, the catalytic domain of a eukaryotic sialyltransferase is expressed in a host cell. Other sialyltransferases that can be used in the invention are found in Table 1 and **FIG. 1**, below.

Table 1

Sialyltransferase	Accession number
ST3Gal I	X73523
ST3Gal II	BC015264
ST3Gal II	X76989
ST3Gal III	BC006710
ST3Gal IV	BC011121
ST3Gal V	AF119416
ST3Gal VI	NM_018784
ST6Gal I	BB768706
ST6Gal I	BB768706
ST6Gal I	D16106
ST6GalNAc I	NM_011371
ST6GalNAc I	NM_011371
ST6GalNAc II	X93999
ST6GalNAc III	Y11342
ST6GalNAc IV	NM_011373
ST6GalNAc IV	Y15779
ST6GalNAc IV	Y15779
ST6GalNAc V	AB028840
ST6GalNAc VI	AB035123
ST6GalNAc VI	AV101836
ST6GalNAc VI	BB772604
ST8Sia I	AW490593
ST8Sia I	NM_011374
ST8Sia II	X83562
ST8Sia II	X83562
ST8Sia III	X80502
ST8Sia IV	X86000
ST8Sia V	X98014
ST8Sia VI	AB059554

[0186] In addition to the sialyltransferases listed in Tables 3 and 4, the invention also includes use of the following sialyltransferases: protein encoded by the siaA protein of *Haemophilus influenzae*, accession number AAL38659; an α 2,6-sialyltransferase gene from *Photobacterium damsela*, accession number BAA25316; protein from *Pasteurella multocida*, accession number NP_245125; and protein from *Haemophilus ducreyi*, accession number NP_872679.

[0262] An example of a sialyltransferase that is useful in the claimed methods is ST3Gal III, which is also referred to as α (2,3)sialyltransferase (EC 2.4.99.6). This enzyme catalyzes the transfer of sialic acid to the Gal of a Gal β 1,3GlcNAc or Gal β 1,4GlcNAc glycoside (see, e.g., Wen *et al.*, *J. Biol. Chem.* **267**: 21011 (1992); Van den Eijnden *et al.*, *J. Biol. Chem.* **256**: 3159 (1991)) and is responsible for sialylation of asparagine-linked oligosaccharides in glycopeptides. The sialic acid is linked to a Gal with the formation of an α -linkage between the two saccharides. Bonding (linkage) between the saccharides is between the 2-position of NeuAc and the 3-position of Gal. This particular enzyme can be isolated from rat liver (Weinstein *et al.*, *J. Biol. Chem.* **257**: 13845 (1982)); the human cDNA (Sasaki *et al.* (1993) *J. Biol. Chem.* **268**: 22782-22787; Kitagawa & Paulson (1994) *J. Biol. Chem.* **269**: 1394-1401) and genomic (Kitagawa *et al.* (1996) *J. Biol. Chem.* **271**: 931-938) DNA sequences are known, facilitating production of this enzyme by recombinant expression. In a preferred embodiment, the claimed sialylation methods use a rat ST3Gal III.

[0263] Other exemplary sialyltransferases of use in the present invention include those isolated from *Campylobacter jejuni*, including the α (2,3). See, e.g., WO99/49051.

[0264] Sialyltransferases other those listed in Table 1, are also useful in an economic and efficient large-scale process for sialylation of commercially important glycopeptides. See, for example, the work of W. Wakarchuk generally and, specifically, U.S. Patent No.s 6,709,834; 6,699,705; 6,689,604; 6,210,933; and 6,096,529; and published U.S. Patent Application No.s 2004/0152165; 2003/0148459; 2002/0042369.

[0265] As a simple test to find out the utility of these other enzymes, various amounts of each enzyme (1-100 mU/mg protein) are reacted with asialo- α_1 AGP (at 1-10 mg/ml) to compare the ability of the sialyltransferase of interest to sialylate glycopeptides relative to either bovine ST6Gal I, ST3Gal III or both sialyltransferases. Alternatively, other glycopeptides or glycopeptides, or N-linked oligosaccharides enzymatically released from the peptide backbone can be used in place of asialo- α_1 AGP for this evaluation.

Sialyltransferases with the ability to sialylate N-linked oligosaccharides of glycopeptides more efficiently than ST6Gal I are useful in a practical large-scale process for peptide sialylation.

GalNAc transferases

5 [0266] N-acetylgalactosaminyltransferases are of use in practicing the present invention, particularly for binding a GalNAc moiety to an amino acid of the O-linked glycosylation site of the peptide. Suitable N-acetylgalactosaminyltransferases include, but are not limited to, α (1,3) N-acetylgalactosaminyltransferase, β (1,4) N-acetylgalactosaminyltransferases (Nagata *et al.*, *J. Biol. Chem.* **267**: 12082-12089 (1992) and Smith *et al.*, *J. Biol. Chem.* **269**: 15162 (1994)) and polypeptide N-acetylgalactosaminyltransferase (Homa *et al.*, *J. Biol. Chem.* **268**: 10 12609 (1993)). See also the work of W. Wakarchuk generally and U.S. Patent No. 6,723,545; and published U.S. Patent Application No. 2003/0180928; 2003/0157658; 2003/0157657; and 2003/0157656.

15 [0267] Production of proteins such as the enzyme GalNAc T_{I-XX} from cloned genes by genetic engineering is well known. See, eg., U.S. Pat. No. 4,761,371. One method involves collection of sufficient samples, then the amino acid sequence of the enzyme is determined by N-terminal sequencing. This information is then used to isolate a cDNA clone encoding a full-length (membrane bound) transferase which upon expression in the insect cell line Sf9 resulted in the synthesis of a fully active enzyme. The acceptor specificity of the enzyme is 20 then determined using a semiquantitative analysis of the amino acids surrounding known glycosylation sites in 16 different proteins followed by in vitro glycosylation studies of synthetic peptides. This work has demonstrated that certain amino acid residues are overrepresented in glycosylated peptide segments and that residues in specific positions surrounding glycosylated serine and threonine residues may have a more marked influence on 25 acceptor efficiency than other amino acid moieties.

Cell-Bound Glycosyltransferases

30 [0268] In another embodiment, the enzymes utilized in the method of the invention are cell-bound glycosyltransferases. Although many soluble glycosyltransferases are known (see, for example, U.S. Pat. No. 5,032,519), glycosyltransferases are generally in membrane-bound form when associated with cells. Many of the membrane-bound enzymes studied thus far are considered to be intrinsic proteins; that is, they are not released from the membranes by sonication and require detergents for solubilization. Surface glycosyltransferases have been identified on the surfaces of vertebrate and invertebrate cells, and it has also been

recognized that these surface transferases maintain catalytic activity under physiological conditions. However, the more recognized function of cell surface glycosyltransferases is for intercellular recognition (Roth, MOLECULAR APPROACHES to SUPRACELLULAR PHENOMENA, 1990).

5 [0269] Methods have been developed to alter the glycosyltransferases expressed by cells. For example, Larsen *et al.*, *Proc. Natl. Acad. Sci. USA* **86**: 8227-8231 (1989), report a genetic approach to isolate cloned cDNA sequences that determine expression of cell surface oligosaccharide structures and their cognate glycosyltransferases. A cDNA library generated from mRNA isolated from a murine cell line known to express UDP-galactose:β-D-
10 galactosyl-1,4-N-acetyl-D-glucosaminide α-1,3-galactosyltransferase was transfected into COS-1 cells. The transfected cells were then cultured and assayed for α 1-3 galactosyltransferase activity.

15 [0270] Francisco *et al.*, *Proc. Natl. Acad. Sci. USA* **89**: 2713-2717 (1992), disclose a method of anchoring β-lactamase to the external surface of *Escherichia coli*. A tripartite fusion consisting of (i) a signal sequence of an outer membrane protein, (ii) a membrane-spanning section of an outer membrane protein, and (iii) a complete mature β-lactamase sequence is produced resulting in an active surface bound β-lactamase molecule. However, the Francisco method is limited only to prokaryotic cell systems and as recognized by the authors, requires the complete tripartite fusion for proper functioning.

20 *Sulfotransferases*

25 [0271] The invention also provides methods for producing peptides that include sulfated molecules, including, for example sulfated polysaccharides such as heparin, heparan sulfate, carragenen, and related compounds. Suitable sulfotransferases include, for example, chondroitin-6-sulphotransferase (chicken cDNA described by Fukuta *et al.*, *J. Biol. Chem.* **270**: 18575-18580 (1995); GenBank Accession No. D49915), glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 1 (Dixon *et al.*, *Genomics* **26**: 239-241 (1995); UL18918), and glycosaminoglycan N-acetylglucosamine N-deacetylase/N-sulphotransferase 2 (murine cDNA described in Orellana *et al.*, *J. Biol. Chem.* **269**: 2270-2276 (1994) and Eriksson *et al.*, *J. Biol. Chem.* **269**: 10438-10443 (1994); human cDNA described in GenBank Accession No. U2304).

Glycosidases

[0272] This invention also encompasses the use of wild-type and mutant glycosidases. Mutant β -galactosidase enzymes have been demonstrated to catalyze the formation of disaccharides through the coupling of an α -glycosyl fluoride to a galactosyl acceptor molecule. (Withers, U.S. Pat. No. 6,284,494; issued Sept. 4, 2001). Other glycosidases of use in this invention include, for example, β -glucosidases, β -galactosidases, β -mannosidases, β -acetyl glucosaminidases, β -N-acetyl galactosaminidases, β -xylosidases, β -fucosidases, cellulases, xylanases, galactanases, mannanases, hemicellulases, amylases, glucoamylases, α -glucosidases, α -galactosidases, α -mannosidases, α -N-acetyl glucosaminidases, α -N-acetyl 10 galactose-aminidases, α -xylosidases, α -fucosidases, and neuraminidases/sialidases.

Immobilized Enzymes

[0273] The present invention also provides for the use of enzymes that are immobilized on a solid and/or soluble support. In an exemplary embodiment, there is provided a glycosyltransferase that is conjugated to a PEG via an intact glycosyl linker according to the 15 methods of the invention. The PEG-linker-enzyme conjugate is optionally attached to solid support. The use of solid supported enzymes in the methods of the invention simplifies the work up of the reaction mixture and purification of the reaction product, and also enables the facile recovery of the enzyme. The glycosyltransferase conjugate is utilized in the methods of the invention. Other combinations of enzymes and supports will be apparent to those of 20 skill in the art.

Enzyme ProductionAcquisition of Enzyme Coding Sequences*General Recombinant Technology*

[0274] This invention relies on routine techniques in the field of recombinant genetics. 25 Basic texts disclosing the general methods of use in this invention include Sambrook and Russell, *Molecular Cloning, A Laboratory Manual* (3rd ed. 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and Ausubel *et al.*, eds., *Current Protocols in Molecular Biology* (1994).

[0275] For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). These 30 are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

[0276] Oligonucleotides that are not commercially available can be chemically synthesized, e.g., according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Lett.* **22**: 1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et. al.*, *Nucleic Acids Res.* **12**: 6159-6168 (1984).

5 Purification of oligonucleotides is performed using any art-recognized strategy, e.g., native acrylamide gel electrophoresis or anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* **255**: 137-149 (1983).

[0277] The sequence of the cloned wild-type enzyme genes, synthetic oligonucleotides, and polynucleotides encoding endoglycoceramide synthases can be verified after cloning using, 10 e.g., the chain termination method for sequencing double-stranded templates of Wallace *et al.*, *Gene* **16**: 21-26 (1981).

Cloning and Subcloning of a Wild-type Enzyme Coding Sequence

[0278] A number of polynucleotide sequences encoding wild-type enzymes, e.g., GenBank Accession No. U39554, have been determined and can be synthesized or obtained from a 15 commercial supplier, such as Blue Heron Biotechnology (Bothell, WA).

[0279] The rapid progress in the studies of human genome has made possible a cloning approach where a human DNA sequence database can be searched for any gene segment that has a certain percentage of sequence homology to a known nucleotide sequence, such as one encoding a previously identified enzyme. Any DNA sequence so identified can be 20 subsequently obtained by chemical synthesis and/or a polymerase chain reaction (PCR) technique such as overlap extension method. For a short sequence, completely *de novo* synthesis may be sufficient; whereas further isolation of full length coding sequence from a human cDNA or genomic library using a synthetic probe may be necessary to obtain a larger gene.

25 [0280] Alternatively, a nucleic acid sequence encoding an enzyme can be isolated from a cDNA or genomic DNA library using standard cloning techniques such as polymerase chain reaction (PCR), where homology-based primers can often be derived from a known nucleic acid sequence encoding an enzyme. Most commonly used techniques for this purpose are described in standard texts, e.g., Sambrook and Russell, *supra*.

30 [0281] cDNA libraries suitable for obtaining a coding sequence for a wild-type enzyme may be commercially available or can be constructed. The general methods of isolating mRNA, making cDNA by reverse transcription, ligating cDNA into a recombinant vector,

transfecting into a recombinant host for propagation, screening, and cloning are well known (see, e.g., Gubler and Hoffman, *Gene*, **25**: 263-269 (1983); Ausubel *et al.*, *supra*). Upon obtaining an amplified segment of nucleotide sequence by PCR, the segment can be further used as a probe to isolate the full length polynucleotide sequence encoding the wild-type 5 enzyme from the cDNA library. A general description of appropriate procedures can be found in Sambrook and Russell, *supra*.

[0282] A similar procedure can be followed to obtain a full length sequence encoding a wild-type enzyme from a genomic library. Genomic libraries are commercially available or can be constructed according to various art-recognized methods. In general, to construct a 10 genomic library, the DNA is first extracted from an organism where an enzyme is likely found. The DNA is then either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb in length. The fragments are subsequently separated by gradient centrifugation from polynucleotide fragments of undesired sizes and are inserted in bacteriophage λ vectors. These vectors and phages are packaged *in vitro*. Recombinant phages are analyzed by plaque 15 hybridization as described in Benton and Davis, *Science*, **196**: 180-182 (1977). Colony hybridization is carried out as described by Grunstein *et al.*, *Proc. Natl. Acad. Sci. USA*, **72**: 3961-3965 (1975).

[0283] Based on sequence homology, degenerate oligonucleotides can be designed as primer sets and PCR can be performed under suitable conditions (see, e.g., White *et al.*, *PCR 20 Protocols: Current Methods and Applications*, 1993; Griffin and Griffin, *PCR Technology*, CRC Press Inc. 1994) to amplify a segment of nucleotide sequence from a cDNA or genomic library. Using the amplified segment as a probe, the full length nucleic acid encoding a wild-type enzyme is obtained.

[0284] Upon acquiring a nucleic acid sequence encoding a wild-type enzyme, the coding 25 sequence can be subcloned into a vector, for instance, an expression vector, so that a recombinant enzyme can be produced from the resulting construct. Further modifications to the wild-type enzyme coding sequence, e.g., nucleotide substitutions, may be subsequently made to alter the characteristics of the enzyme.

Introducing Mutations into the Enzyme Coding Sequence

[0285] Modifications altering the enzymatic activity of an enzyme may be made in various 30 locations within the polynucleotide coding sequence. The preferred locations for such modifications are, however, within the active site of the enzyme. A conserved sequence

encoding a three-amino acid segment Asn-Glu-Pro was previously identified within the active site of enzymes, and the Glu residue within the segment appears to be connected to the activity of the enzyme (Sakaguchi *et al.*, *Biochem. Biophys. Res. Commun.*, 1999, **260**: 89-93)

5 [0286] From an encoding nucleic acid sequence, the amino acid sequence of a wild-type enzyme, *e.g.*, SEQ ID NO:1 or 2, can be deduced and the presence of an active site can be confirmed. Preferably, mutations are introduced into the active site. For instance, the Glu residue at position 233 of SEQ ID NO:1 or position 224 of SEQ ID NO:2, both located in the middle of a three-amino acid segment Asn-Glu-Pro, can be targeted for mutation, such as
10 deletion or substitution by another amino acid residue. In addition, other Glu residues, *e.g.*, the Glu located at position 351 of SEQ ID NO:1 or position 343 of SEQ ID NO:2, are also targets for introducing mutations to alter the enzymatic activity of an enzyme. An artisan can accomplish the goal of mutating a target Glu residue by employing any one of the well known mutagenesis methods, which are discussed in detail below. Exemplary modifications
15 are introduced to replace the Glu residue with another amino acid residue as depicted in SEQ ID NOs:3-7.

[0287] A variety of diversity-generating protocols are established and described in the art. *See, e.g.*, Zhang *et al.*, *Proc. Natl. Acad. Sci. USA*, **94**: 4504-4509 (1997); and Stemmer, *Nature*, **370**: 389-391 (1994). The procedures can be used separately or in combination to
20 produce variants of a set of nucleic acids, and hence variants of encoded polypeptides. Kits for mutagenesis, library construction, and other diversity-generating methods are commercially available.

[0288] Mutational methods of generating diversity include, for example, site-directed mutagenesis (Botstein and Shortle, *Science*, **229**: 1193-1201 (1985)), mutagenesis using
25 uracil-containing templates (Kunkel, *Proc. Natl. Acad. Sci. USA*, **82**: 488-492 (1985)), oligonucleotide-directed mutagenesis (Zoller and Smith, *Nucl. Acids Res.*, **10**: 6487-6500 (1982)), phosphorothioate-modified DNA mutagenesis (Taylor *et al.*, *Nucl. Acids Res.*, **13**: 8749-8764 and 8765-8787 (1985)), and mutagenesis using gapped duplex DNA (Kramer *et al.*, *Nucl. Acids Res.*, **12**: 9441-9456 (1984)).

30 [0289] Other possible methods for generating mutations include point mismatch repair (Kramer *et al.*, *Cell*, **38**: 879-887 (1984)), mutagenesis using repair-deficient host strains (Carter *et al.*, *Nucl. Acids Res.*, **13**: 4431-4443 (1985)), deletion mutagenesis (Eghtedarzadeh

and Henikoff, *Nucl. Acids Res.*, **14**: 5115 (1986)), restriction-selection and restriction-purification (Wells *et al.*, *Phil. Trans. R. Soc. Lond. A*, **317**: 415-423 (1986)), mutagenesis by total gene synthesis (Nambiar *et al.*, *Science*, **223**: 1299-1301 (1984)), double-strand break repair (Mandecki, *Proc. Natl. Acad. Sci. USA*, **83**: 7177-7181 (1986)), mutagenesis by 5 polynucleotide chain termination methods (U.S. Patent No. 5,965,408), and error-prone PCR (Leung *et al.*, *Biotechniques*, **1**: 11-15 (1989)).

[0290] At the completion of modification, the mutant enzyme coding sequences can then be subcloned into an appropriate vector for recombinant production in the same manner as the wild-type genes.

10 *Modification of Nucleic Acids for Preferred Codon Usage in a Host Organism*

[0291] The polynucleotide sequence encoding an enzyme (either wild-type or mutant) can be altered to coincide with the preferred codon usage of a particular host. For example, the preferred codon usage of one strain of bacteria can be used to derive a polynucleotide that encodes a mutant enzyme of the invention and includes the codons favored by this strain.

15 The frequency of preferred codon usage exhibited by a host cell can be calculated by averaging frequency of preferred codon usage in a large number of genes expressed by the host cell (e.g., calculation service is available from web site of the Kazusa DNA Research Institute, Japan). This analysis is preferably limited to genes that are highly expressed by the host cell. U.S. Patent No. 5,824,864, for example, provides the frequency of codon usage by 20 highly expressed genes exhibited by dicotyledonous plants and monocotyledonous plants.

[0292] The sequences of the cloned enzyme genes, synthetic polynucleotides, and modified enzyme genes can be verified using, e.g., the chain termination method for sequencing double-stranded templates as described in Wallace *et al.*, *Gene* **16**:21-26 (1981).

Expression and Purification of the Enzymes

25 [0293] Following sequence verification, the wild-type or mutant enzyme of the present invention can be produced using routine techniques in the field of recombinant genetics, relying on the polynucleotide sequences encoding the polypeptide disclosed herein.

Expression Systems

[0294] To obtain high level expression of a nucleic acid encoding a wild-type or a mutant 30 enzyme of the present invention, one typically subclones a polynucleotide encoding the enzyme into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator and a ribosome binding site for translational initiation.

Suitable bacterial promoters are well known in the art and described, *e.g.*, in Sambrook and Russell, *supra*, and Ausubel *et al.*, *supra*. Bacterial expression systems for expressing the wild-type or mutant enzyme are available in, *e.g.*, *E. coli*, *Bacillus sp.*, *Salmonella*, and *Caulobacter*. Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. In one embodiment, the eukaryotic expression vector is an adenoviral vector, an adeno-associated vector, or a retroviral vector.

5 [0295] The promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is optionally positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

10 [0296] In addition to the promoter, the expression vector typically includes a transcription unit or expression cassette that contains all the additional elements required for the expression of the enzyme in host cells. A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence encoding the wild-type or mutant enzyme and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. The nucleic acid sequence encoding the enzyme is typically linked to a cleavable signal peptide sequence to promote secretion of the enzyme by 15 the transformed cell. Such signal peptides include, among others, the signal peptides from tissue plasminogen activator, insulin, and neuron growth factor, and juvenile hormone esterase of *Heliothis virescens*. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

20 [0297] In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

25 [0298] The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include 30 plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems

such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, *e.g.*, c-myc.

[0299] Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, *e.g.*, SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A⁺, pMTO10/A⁺, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[0300] Some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase. Alternatively, high yield expression systems not involving gene amplification are also suitable, such as a baculovirus vector in insect cells, with a polynucleotide sequence encoding the mutant enzyme under the direction of the polyhedrin promoter or other strong baculovirus promoters.

[0301] The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are optionally chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary.

[0302] As discussed above, a person skilled in the art will recognize that various conservative substitutions can be made to any wild-type or mutant enzyme or its coding sequence while still retaining the synthetic activity of the enzyme. Moreover, modifications of a polynucleotide coding sequence may also be made to accommodate preferred codon usage in a particular expression host without altering the resulting amino acid sequence.

Transfection Methods

[0303] Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of the wild-type or mutant enzyme, which are then purified using standard techniques (*see, e.g.*, Colley *et al.*, *J. Biol. Chem.* **264**: 17619-

17622 (1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (see, e.g., Morrison, *J. Bact.* **132**: 349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* **101**: 347-362 (Wu *et al.*, eds, 1983).

5 [0304] Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA, or other foreign genetic material into a host cell (see, e.g., Sambrook and 10 Russell, *supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing the wild-type or mutant enzyme.

Detection of the Expression of Recombinant Enzymes

15 [0305] After the expression vector is introduced into appropriate host cells, the transfected cells are cultured under conditions favoring expression of the wild-type or mutant enzyme. The cells are then screened for the expression of the recombinant polypeptide, which is subsequently recovered from the culture using standard techniques (see, e.g., Scopes, *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al.*, *supra*; and Sambrook and Russell, *supra*).

20 [0306] Several general methods for screening gene expression are well known among those skilled in the art. First, gene expression can be detected at the nucleic acid level. A variety of methods of specific DNA and RNA measurement using nucleic acid hybridization techniques are commonly used (e.g., Sambrook and Russell, *supra*). Some methods involve an electrophoretic separation (e.g., Southern blot for detecting DNA and Northern blot for 25 detecting RNA), but detection of DNA or RNA can be carried out without electrophoresis as well (such as by dot blot). The presence of nucleic acid encoding an enzyme in transfected cells can also be detected by PCR or RT-PCR using sequence-specific primers.

30 [0307] Second, gene expression can be detected at the polypeptide level. Various immunological assays are routinely used by those skilled in the art to measure the level of a gene product, particularly using polyclonal or monoclonal antibodies that react specifically with a wild-type or mutant enzyme of the present invention, such as a polypeptide having the amino acid sequence of SEQ ID NO:3, 4, or 5, (e.g., Harlow and Lane, *Antibodies, A*

Laboratory Manual, Chapter 14, Cold Spring Harbor, 1988; Kohler and Milstein, *Nature*, **256**: 495-497 (1975)). Such techniques require antibody preparation by selecting antibodies with high specificity against the recombinant polypeptide or an antigenic portion thereof. The methods of raising polyclonal and monoclonal antibodies are well established and their descriptions can be found in the literature, *see, e.g.*, Harlow and Lane, *supra*; Kohler and Milstein, *Eur. J. Immunol.*, **6**: 511-519 (1976). More detailed descriptions of preparing antibody against the mutant enzyme of the present invention and conducting immunological assays detecting the mutant enzyme are provided in a later section.

5 [0308] In addition, functional assays may also be performed for the detection of a recombinant enzyme in transfected cells. Assays for detecting hydrolytic or synthetic activity 10 of the recombinant enzyme are generally described in a later section.

Purification of Recombinant Enzymes

15 [0309] Once the expression of a recombinant enzyme in transfected host cells is confirmed, the host cells are then cultured in an appropriate scale for the purpose of purifying the recombinant enzyme.

Purification of Recombinant Polypeptides from Bacteria

20 [0310] When the enzymes of the present invention are produced recombinantly by transformed bacteria in large amounts, typically after promoter induction, although expression can be constitutive, the proteins may form insoluble aggregates. There are several protocols that are suitable for purification of protein inclusion bodies. For example, 25 purification of aggregate proteins (hereinafter referred to as inclusion bodies) typically involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, *e.g.*, by incubation in a buffer of about 100-150 µg/ml lysozyme and 0.1% Nonidet P40, a non-ionic detergent. The cell suspension can be ground using a Polytron grinder (Brinkman Instruments, Westbury, NY). Alternatively, the cells can be sonicated on ice. Alternate methods of lysing bacteria are described in Ausubel *et al.* and Sambrook and Russell, both *supra*, and will be apparent to those of skill in the art.

30 [0311] The cell suspension is generally centrifuged and the pellet containing the inclusion bodies resuspended in buffer which does not dissolve but washes the inclusion bodies, *e.g.*, 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 150 mM NaCl and 2% Triton-X 100, a non-ionic detergent. It may be necessary to repeat the wash step to remove as much cellular debris as possible. The remaining pellet of inclusion bodies may be resuspended in an appropriate

buffer (*e.g.*, 20 mM sodium phosphate, pH 6.8, 150 mM NaCl). Other appropriate buffers will be apparent to those of skill in the art.

[0312] Following the washing step, the inclusion bodies are solubilized by the addition of a solvent that is both a strong hydrogen acceptor and a strong hydrogen donor (or a combination of solvents each having one of these properties). The proteins that formed the inclusion bodies may then be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to, urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Some solvents that are capable of solubilizing aggregate-forming proteins, such as SDS (sodium dodecyl sulfate) and 70% formic acid, may be inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a lack of immunogenicity and/or activity. Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of the immunologically and/or biologically active protein of interest. After solubilization, the protein can be separated from other bacterial proteins by standard separation techniques.

[0313] Alternatively, it is possible to purify recombinant polypeptides, *e.g.*, a mutant enzyme, from bacterial periplasm. Where the recombinant protein is exported into the periplasm of the bacteria, the periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to those of skill in the art (*see e.g.*, Ausubel *et al.*, *supra*). To isolate recombinant proteins from the periplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM MgSO₄ and kept in an ice bath for approximately 10 minutes. The cell suspension is centrifuged and the supernatant decanted and saved. The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

Standard Protein Separation Techniques For Purification

[0314] When a recombinant polypeptide, *e.g.*, the mutant enzyme of the present invention, is expressed in host cells in a soluble form, its purification can follow the standard protein purification procedure described below.

Solubility Fractionation

[0315] Often as an initial step, and if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest, *e.g.*, a mutant enzyme of the 5 present invention. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol is to add saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate 10 concentration is between 20-30%. This will precipitate the most hydrophobic proteins. The precipitate is discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, through either dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold 15 ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

Size Differential Filtration

[0316] Based on a calculated molecular weight, a protein of greater and lesser size can be isolated using ultrafiltration through membranes of different pore sizes (for example, Amicon 20 or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of a protein of interest, *e.g.*, a mutant enzyme. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant protein will pass through the membrane 25 into the filtrate. The filtrate can then be chromatographed as described below.

Column Chromatography

[0317] The proteins of interest (such as the mutant enzyme of the present invention) can also be separated from other proteins on the basis of their size, net surface charge, 30 hydrophobicity, or affinity for ligands. In addition, antibodies raised against enzyme can be conjugated to column matrices and the enzyme immunopurified. All of these methods are well known in the art.

[0318] It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (e.g., Pharmacia Biotech).

Enzyme Assays

5 *Production of Antibodies against Enzymes and Immunoassays for Detection of Enzyme Expression*

[0319] To confirm the production of a recombinant enzyme, immunological assays may be useful to detect in a sample the expression of the enzyme. Immunological assays are also useful for quantifying the expression level of the recombinant enzyme.

10 *Production of Antibodies against Enzyme*

[0320] Methods for producing polyclonal and monoclonal antibodies that react specifically with an immunogen of interest are known to those of skill in the art (see, e.g., Coligan, *Current Protocols in Immunology* Wiley/Greene, NY, 1991; Harlow and Lane, *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY, 1989; Stites *et al.* (eds.) *Basic and*

15 *Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY, 1986; and Kohler and Milstein *Nature* **256**: 495-497, 1975). Such techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors (see, Huse *et al.*, *Science* **246**: 1275-1281, 20 1989; and Ward *et al.*, *Nature* **341**: 544-546, 1989).

[0321] In order to produce antisera containing antibodies with desired specificity, the polypeptide of interest (e.g., a mutant enzyme of the present invention) or an antigenic fragment thereof can be used to immunize suitable animals, e.g., mice, rabbits, or primates. A standard adjuvant, such as Freund's adjuvant, can be used in accordance with a standard 25 immunization protocol. Alternatively, a synthetic antigenic peptide derived from that particular polypeptide can be conjugated to a carrier protein and subsequently used as an immunogen.

[0322] The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the antigen of interest. When 30 appropriately high titers of antibody to the antigen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich antibodies specifically reactive to the antigen and purification of the antibodies can be performed

subsequently, *see*, Harlow and Lane, *supra*, and the general descriptions of protein purification provided above.

[0323] Monoclonal antibodies are obtained using various techniques familiar to those of skill in the art. Typically, spleen cells from an animal immunized with a desired antigen are 5 immortalized, commonly by fusion with a myeloma cell (*see*, Kohler and Milstein, *Eur. J. Immunol.* **6**:511-519, 1976). Alternative methods of immortalization include, *e.g.*, transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and the yield of the 10 monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host.

[0324] Additionally, monoclonal antibodies may also be recombinantly produced upon identification of nucleic acid sequences encoding an antibody with desired specificity or a binding fragment of such antibody by screening a human B cell cDNA library according to 15 the general protocol outlined by Huse *et al.*, *supra*. The general principles and methods of recombinant polypeptide production discussed above are applicable for antibody production by recombinant methods.

[0325] When necessary, antibodies capable of specifically recognizing a mutant enzyme of 20 the present invention can be tested for their cross-reactivity against the corresponding wild-type enzyme and thus distinguished from the antibodies against the wild-type enzyme. For instance, antisera obtained from an animal immunized with a mutant enzyme can be run through a column on which a corresponding wild-type enzyme is immobilized. The portion 25 of the antisera that passes through the column recognizes only the mutant enzyme and not the corresponding wild-type enzyme. Similarly, monoclonal antibodies against a mutant enzyme can also be screened for their exclusivity in recognizing only the mutant but not the wild-type enzyme.

[0326] Polyclonal or monoclonal antibodies that specifically recognize only the mutant 30 enzyme of the present invention but not the corresponding wild-type enzyme are useful for isolating the mutant enzyme from the wild-type enzyme, for example, by incubating a sample with a mutant enzyme-specific polyclonal or monoclonal antibody immobilized on a solid support.

Immunoassays for Detecting Enzyme Expression

[0327] Once antibodies specific for an enzyme of the present invention are available, the amount of the polypeptide in a sample, *e.g.*, a cell lysate, can be measured by a variety of immunoassay methods providing qualitative and quantitative results to a skilled artisan. For 5 a review of immunological and immunoassay procedures in general *see, e.g.*, Stites, *supra*; U.S. Patent Nos. 4,366,241; 4,376,110; 4,517,288; and 4,837,168.

Labeling in Immunoassays

[0328] Immunoassays often utilize a labeling agent to specifically bind to and label the binding complex formed by the antibody and the target protein. The labeling agent may itself 10 be one of the moieties comprising the antibody/target protein complex, or may be a third moiety, such as another antibody, that specifically binds to the antibody/target protein complex. A label may be detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Examples include, but are not limited to, magnetic beads (*e.g.*, DynabeadsTM), fluorescent dyes (*e.g.*, fluorescein 15 isothiocyanate, Texas red, rhodamine, and the like), radiolabels (*e.g.*, ³H, ¹²⁵I, ³⁵S, ¹⁴C, or ³²P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase, and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.*, polystyrene, polypropylene, latex, *etc.*) beads.

[0329] In some cases, the labeling agent is a second antibody bearing a detectable label.

20 Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second antibody can be modified with a detectable moiety, such as biotin, to which a third labeled molecule can specifically bind, such as enzyme-labeled streptavidin.

[0330] Other proteins capable of specifically binding immunoglobulin constant regions, 25 such as protein A or protein G, can also be used as the label agents. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (*see, generally*, Kronval, et al. *J. Immunol.*, **111**: 1401-1406 (1973); and Akerstrom, et al., *J. Immunol.*, **135**: 2589-2542 (1985)).

Immunoassay Formats

[0331] Immunoassays for detecting a target protein of interest (*e.g.*, a recombinant enzyme) from samples may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured target protein is directly measured. In one

preferred “sandwich” assay, for example, the antibody specific for the target protein can be bound directly to a solid substrate where the antibody is immobilized. It then captures the target protein in test samples. The antibody/target protein complex thus immobilized is then bound by a labeling agent, such as a second or third antibody bearing a label, as described 5 above.

[0332] In competitive assays, the amount of target protein in a sample is measured indirectly by measuring the amount of an added (exogenous) target protein displaced (or 10 competed away) from an antibody specific for the target protein by the target protein present in the sample. In a typical example of such an assay, the antibody is immobilized and the exogenous target protein is labeled. Since the amount of the exogenous target protein bound to the antibody is inversely proportional to the concentration of the target protein present in the sample, the target protein level in the sample can thus be determined based on the amount of exogenous target protein bound to the antibody and thus immobilized.

[0333] In some cases, western blot (immunoblot) analysis is used to detect and quantify the 15 presence of a wild-type or mutant enzyme in the samples. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support (such as a nitrocellulose filter, a nylon filter, or a derivatized nylon filter) and incubating the samples with the antibodies that specifically bind the target protein. These antibodies may be directly labeled or alternatively 20 may be subsequently detected using labeled antibodies (*e.g.*, labeled sheep anti-mouse antibodies) that specifically bind to the antibodies against the enzyme.

[0334] Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (*see*, 25 Monroe et al., *Amer. Clin. Prod. Rev.*, **5**: 34-41 (1986)).

Fusion Proteins

[0335] In other exemplary embodiments, the methods of the invention utilize fusion proteins that have more than one enzymatic activity that is involved in synthesis of a desired glycopeptide conjugate. The fusion polypeptides can be composed of, for example, a 30 catalytically active domain of a glycosyltransferase that is joined to a catalytically active domain of an accessory enzyme. The accessory enzyme catalytic domain can, for example, catalyze a step in the formation of a nucleotide sugar that is a donor for the

glycosyltransferase, or catalyze a reaction involved in a glycosyltransferase cycle. For example, a polynucleotide that encodes a glycosyltransferase can be joined, in-frame, to a polynucleotide that encodes an enzyme involved in nucleotide sugar synthesis. The resulting fusion protein can then catalyze not only the synthesis of the nucleotide sugar, but also the transfer of the sugar moiety to the acceptor molecule. The fusion protein can be two or more cycle enzymes linked into one expressible nucleotide sequence. In other embodiments the fusion protein includes the catalytically active domains of two or more glycosyltransferases. See, for example, 5,641,668. The modified glycopeptides of the present invention can be readily designed and manufactured utilizing various suitable fusion proteins (see, for example, PCT Patent Application PCT/CA98/01180, which was published as WO 99/31224 on June 24, 1999.)

Purification of Peptide- and Other-Conjugates

[0336] The products produced by the above processes can be used without purification. However, it is usually preferred to recover the product. Standard, well-known techniques for recovery of glycosylated saccharides such as thin or thick layer chromatography, column chromatography, ion exchange chromatography, or membrane filtration can be used. It is preferred to use membrane filtration, more preferably utilizing a reverse osmotic membrane, or one or more column chromatographic techniques for the recovery as is discussed hereinafter and in the literature cited herein. For instance, membrane filtration wherein the membranes have molecular weight cutoff of about 3000 to about 10,000 can be used to remove proteins such as glycosyl transferases. Nanofiltration or reverse osmosis can then be used to remove salts and/or purify the product saccharides (see, e.g., WO 98/15581). Nanofilter membranes are a class of reverse osmosis membranes that pass monovalent salts but retain polyvalent salts and uncharged solutes larger than about 100 to about 2,000 Daltons, depending upon the membrane used. Thus, in a typical application, saccharides prepared by the methods of the present invention will be retained in the membrane and contaminating salts will pass through.

[0337] If the modified glycoprotein is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration; optionally, the protein may be concentrated with a commercially available protein concentration filter, followed by separating the polypeptide variant from other impurities by one or more steps selected from immunoaffinity

chromatography, ion-exchange column fractionation (*e.g.*, on diethylaminoethyl (DEAE) or matrices containing carboxymethyl or sulfopropyl groups), chromatography on Blue-Sepharose, CM Blue-Sepharose, MONO-Q, MONO-S, lentil lectin-Sepharose, WGA-Sepharose, Con A-Sepharose, Ether Toyopearl, Butyl Toyopearl, Phenyl Toyopearl, SP-Sepharose, or protein A Sepharose, SDS-PAGE chromatography, silica chromatography, chromatofocusing, reverse phase HPLC (*e.g.*, silica gel with appended aliphatic groups), gel filtration using, *e.g.*, Sephadex molecular sieve or size-exclusion chromatography, chromatography on columns that selectively bind the polypeptide, and ethanol or ammonium sulfate precipitation.

5 [0338] Modified glycopeptides produced in culture are usually isolated by initial extraction from cells, enzymes, etc., followed by one or more concentration, salting-out, aqueous ion-exchange, or size-exclusion chromatography steps, *e.g.*, SP Sepharose. Additionally, the modified glycoprotein may be purified by affinity chromatography. HPLC may also be employed for one or more purification steps.

10 [0339] A protease inhibitor, *e.g.*, methylsulfonylfluoride (PMSF) may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

15 [0340] Within another embodiment, supernatants from systems which produce the modified glycopeptide of the invention are first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate may be applied to a suitable purification matrix. For example, a suitable affinity matrix may comprise a ligand for the peptide, a lectin or antibody molecule bound to a suitable support. Alternatively, an anion-exchange resin may be employed, for example, a matrix or substrate having pendant DEAE groups. Suitable 20 matrices include acrylamide, agarose, dextran, cellulose, or other types commonly employed in protein purification. Alternatively, a cation-exchange step may be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are particularly preferred.

25 [0341] Finally, one or more RP-HPLC steps employing hydrophobic RP-HPLC media, *e.g.*, silica gel having pendant methyl or other aliphatic groups, may be employed to further purify a polypeptide variant composition. Some or all of the foregoing purification steps, in various 30 combinations, can also be employed to provide a homogeneous modified glycoprotein.

[0342] The modified glycopeptide of the invention resulting from a large-scale fermentation may be purified by methods analogous to those disclosed by Urdal *et al.*, *J. Chromatog.* **296**: 171 (1984). This reference describes two sequential, RP-HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column. Alternatively, 5 techniques such as affinity chromatography may be utilized to purify the modified glycoprotein.

[0343] In another aspect, the invention provides a pharmaceutical composition. The pharmaceutical composition includes a pharmaceutically acceptable diluent and a covalent conjugate between a substrate (peptide, glycolipid, aglycone, etc.) and a modified sugar of 10 the invention.

[0344] An exemplary conjugate is formed between a non-naturally-occurring, water-soluble polymer, therapeutic moiety or biomolecule and a glycosylated or non-glycosylated peptide. The polymer, therapeutic moiety or biomolecule is conjugated to the peptide via an intact glycosyl linking group interposed between and covalently linked to both the peptide 15 and the polymer, therapeutic moiety or biomolecule.

[0345] Pharmaceutical compositions of the invention are suitable for use in a variety of drug delivery systems. Suitable formulations for use in the present invention are found in *Remington's Pharmaceutical Sciences*, Mace Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, *see*, Langer, *Science* **249**:1527-20 1533 (1990).

[0346] The pharmaceutical compositions may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a 25 wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable matrices, such as microspheres (*e.g.*, polylactate polyglycolate), may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are 30 disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109.

[0347] Commonly, the pharmaceutical compositions are administered subcutaneously or parenterally, *e.g.*, intravenously. Thus, the invention provides compositions for parenteral

administration which comprise the compound dissolved or suspended in an acceptable carrier, preferably an aqueous carrier, *e.g.*, water, buffered water, saline, PBS and the like. The compositions may also contain detergents such as Tween 20 and Tween 80; stabilizers such as mannitol, sorbitol, sucrose, and trehalose; and preservatives such as EDTA and m-cresol. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents and the like.

5 [0348] These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or 10 lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably from 5 to 9 and most preferably from 7 and 8.

15 [0349] In some embodiments the glycopeptides of the invention can be incorporated into liposomes formed from standard vesicle-forming lipids. A variety of methods are available for preparing liposomes, as described in, *e.g.*, Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* **9**: 467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728 and 4,837,028. The targeting of liposomes using a variety of targeting agents (*e.g.*, the sialyl galactosides of the invention) is well known in the art (*see, e.g.*, U.S. Patent Nos. 4,957,773 and 4,603,044).

20 [0350] Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes of lipid components, such as phosphatidylethanolamine, which can be activated for attachment of targeting agents, or derivatized lipophilic compounds, such as lipid-derivatized glycopeptides of the invention.

25 [0351] Targeting mechanisms generally require that the targeting agents be positioned on the surface of the liposome in such a manner that the target moieties are available for interaction with the target, for example, a cell surface receptor. The carbohydrates of the invention may be attached to a lipid molecule before the liposome is formed using methods known to those of skill in the art (*e.g.*, alkylation or acylation of a hydroxyl group present on the carbohydrate with a long chain alkyl halide or with a fatty acid, respectively).

30 Alternatively, the liposome may be fashioned in such a way that a connector portion is first incorporated into the membrane at the time of forming the membrane. The connector portion must have a lipophilic portion, which is firmly embedded and anchored in the membrane. It must also have a reactive portion, which is chemically available on the aqueous surface of the

liposome. The reactive portion is selected so that it will be chemically suitable to form a stable chemical bond with the targeting agent or carbohydrate, which is added later. In some cases it is possible to attach the target agent to the connector molecule directly, but in most instances it is more suitable to use a third molecule to act as a chemical bridge, thus linking 5 the connector molecule which is in the membrane with the target agent or carbohydrate which is extended, three dimensionally, off of the vesicle surface.

[0352] The compounds prepared by the methods of the invention may also find use as diagnostic reagents. For example, labeled compounds can be used to locate areas of inflammation or tumor metastasis in a patient suspected of having an inflammation. For this 10 use, the compounds can be labeled with ^{125}I , ^{14}C , or tritium.

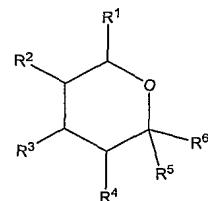
[0353] The following examples are provided to illustrate the conjugates, and methods and of the present invention, but not to limit the claimed invention.

[0354] While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others 15 skilled in the art without departing from the true spirit and scope of the invention.

[0355] All patents, patent applications, and other publications cited in this application are incorporated by reference in the entirety.

WHAT IS CLAIMED IS:

1 1. A peptide conjugate comprising the glycosyl moiety:



2 2 wherein

3 3 R¹, R², R³, R⁴, and R⁵ are members independently selected from H, OR^{7a}, N(R^{7a})₂,
 4 4 SR^{7a}, JC(O)R⁷, substituted or unsubstituted alkyl, substituted or unsubstituted
 5 5 heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted
 6 6 heteroaryl or substituted or unsubstituted heterocycloalkyl

7 7 wherein

8 8 R⁷ is a member selected from H, OR⁸, NR⁸R⁹, substituted or unsubstituted
 9 9 alkyl, substituted or unsubstituted heteroalkyl, substituted or
 10 10 unsubstituted aryl, substituted or unsubstituted heteroaryl, or
 11 11 substituted or unsubstituted heterocycloalkyl

12 12 wherein

13 13 R⁸ and R⁹ are member independently selected from H, substituted or
 14 14 unsubstituted alkyl, substituted or unsubstituted heteroalkyl,
 15 15 substituted or unsubstituted aryl, substituted or unsubstituted
 16 16 heteroaryl, and substituted or unsubstituted heterocycloalkyl

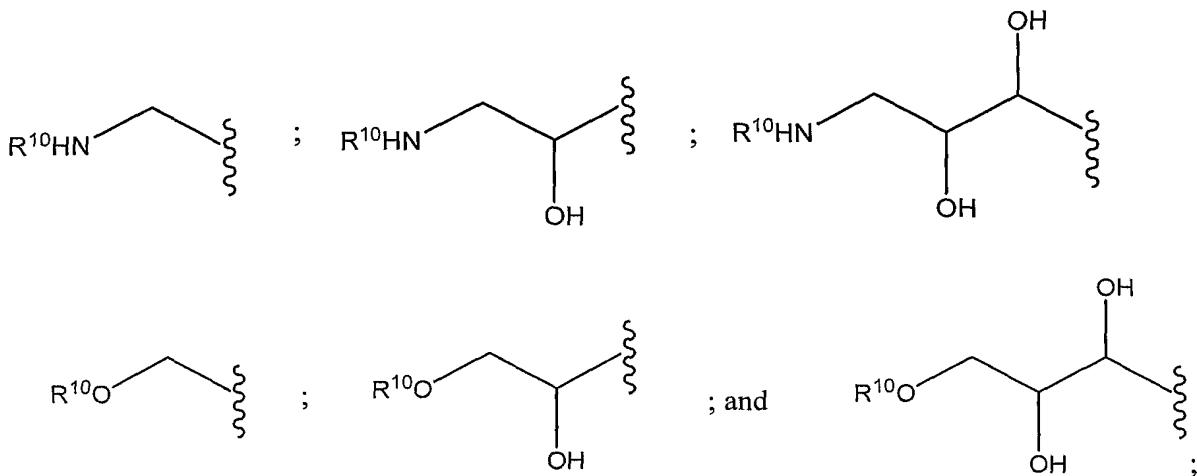
17 17 R^{7a} is a member selected from H, substituted or unsubstituted alkyl,
 18 18 substituted or unsubstituted heteroalkyl, substituted or unsubstituted
 19 19 aryl, substituted or unsubstituted heteroaryl, or substituted or
 20 20 unsubstituted heterocycloalkyl

21 21 J is a member selected from a bond, O, S or NH,
 22 22 at least one of R¹, R², R³, R⁴, and R⁵ comprises a polymer moiety linked
 23 23 through an acyl group; and

24 24 R⁶ is a member selected from an amino acid residue of said peptide, a carbohydrate
 25 25 linker moiety covalently bound to an amino acid residue of said peptide, and
 26 26 combinations thereof.

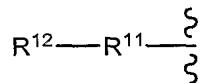
1 2. The conjugate according to claim 1 wherein

2 R¹ is a member selected from:



5 and R¹⁰ comprises a modifying group.

1 4. The conjugate according to claim 1 wherein said modifying group is a
2 member selected from poly(ether), poly(sialic acid), and poly(amino acid).

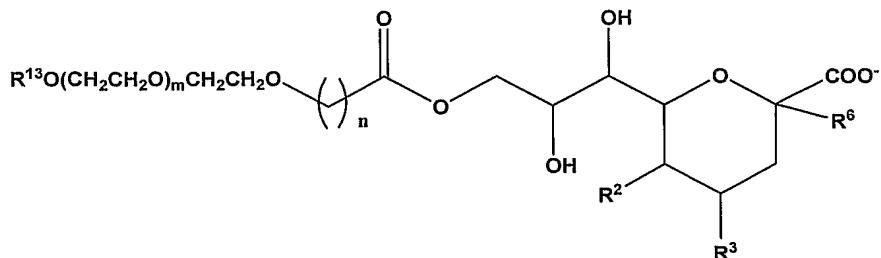


3 wherein

4 said linker is R¹¹, and R¹¹ is a member selected from substituted or unsubstituted alkyl
5 and substituted or unsubstituted heteroalkyl;
6 said modifying group is R¹²; and
7 ~~~~ represents a connection to the remainder of the conjugate through a member
8 selected from O and N.

1 6. The conjugate according to claim 5, wherein R¹¹ is an acyl moiety,
2 such that said acyl moiety, taken together with the atoms to which it is covalently attached,
3 comprises a moiety selected from an ester, amide and urethane.

1 7. The conjugate according to claim 1 having the structure:



2

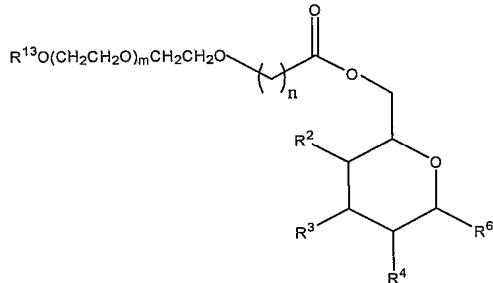
3 in which

4 m is an integer from 1 to 2500;

5 n is an integer from 0 to 40; and

6 R¹³ is a member selected from H and substituted or unsubstituted alkyl.

1 8. The conjugate according to claim 1 having the structure:



2

3 in which

4 m is an integer from 1 to 2500;

5 n is an integer from 0 to 40; and

6 R¹³ is a member selected from H and substituted or unsubstituted alkyl.

1 9. The conjugate according to claim 8 in which a member selected from

2 R³ and R⁴ is N-acetyl.

1 10. The conjugate according to claim 8 wherein R⁶ is a member selected

2 from Gal, GalNAc, Man, GlcNAc and Glu.

1 11. A method of preparing a conjugate according to claim 1, said method
2 comprising:

3 (a) contacting a peptide comprising a glycosyl residue with

4 (i) an acylating agent comprising an activated acyl moiety; and

(ii) an enzyme for which said acylating agent is a substrate,
under conditions appropriate to acylate said glycosyl residue,
thereby preparing said conjugate.

1 **12.** The method according to claim **11**, wherein said glycosyl residue is
2 acylated by said acylating agent at a site that is a member selected from an OH, NH₂ and SH.

1 **13.** The method according to claim **11**, wherein said enzyme is a member
2 selected from a lipase, a protease, an esterase, an acylase and an acyltransferase.

1 **14.** The method according to claim **11**, wherein said enzyme has an amino
2 acid sequence that is a wild-type sequence for said enzyme.

1 **16.** The method according to claim 15, wherein said mutated enzyme has
2 an acylation activity that is enhanced relative to a corresponding wild-type enzyme.

1 **17.** The method according to claim **15**, wherein said mutated amino acid
2 sequence comprises a mutation wherein an amino acid residue, implicated in hydrolysis of a
3 member selected from an amide and an ester, is replaced by an amino acid residue that is not
4 implicated in said hydrolysis.

FIGURE 1A

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
At1g08280	<i>Arabidopsis thaliana</i>	n.d.	AC011438 BT004583 NC_003070	AAF18241.1 AAO42829.1 NP_172305.1	Q84W00 Q9SGD2	
At1g08660/F22O13.14	<i>Arabidopsis thaliana</i>	n.d.	AC003981 AY064135 AY124807 NC_003070	AAF99778.1 AAL36042.1 AAM70516.1 NP_172342.1	Q8VZJ0 Q9FRR9	
At3g48820/T21J18_90	<i>Arabidopsis thaliana</i>	n.d.	AY080589 AY133816 AL132963 NM_114741	AAL85966.1 AAM91750.1 CAB87910.1 NP_190451.1	Q8RY00 Q9M301	
α -2,3-sialyltransferase (ST3GAL-IV)	<i>Bos taurus</i>	n.d.	AJ584673	CAE48298.1		
α -2,3-sialyltransferase (St3Gal-V)	<i>Bos taurus</i>	n.d.	AJ585768	CAE51392.1		
α -2,6-sialyltransferase (Siat7b)	<i>Bos taurus</i>	n.d.	AJ620651	CAF05850.1		
α -2,8-sialyltransferase (SIAT8A)	<i>Bos taurus</i>	2.4.99.8	AJ699418	CAG27880.1		
α -2,8-sialyltransferase (Siat8D)	<i>Bos taurus</i>	n.d.	AJ699421	CAG27883.1		
α -2,8-sialyltransferase ST8Sia-III (Siat8C)	<i>Bos taurus</i>	n.d.	AJ704563	CAG28696.1		
CMP α -2,6-sialyltransferase (ST6Gal I)	<i>Bos taurus</i>	2.4.99.1	Y15111 NM_177517	CAA75385.1 NP_803483.1	O18974	
sialyltransferase 8 (fragment)	<i>Bos taurus</i>	n.d.	AF450088	AAL47018.1	Q8WN13	
sialyltransferase ST3Gal-II (Siat4B)	<i>Bos taurus</i>	n.d.	AJ748841	CAG44450.1		
sialyltransferase ST3Gal-III (Siat6)	<i>Bos taurus</i>	n.d.	AJ748842	CAG44451.1		
sialyltransferase ST3Gal-VI (Siat10)	<i>Bos taurus</i>	n.d.	AJ748843	CAG44452.1		
ST3Gal I	<i>Bos taurus</i>	n.d.	AJ305086	CAC24698.1	Q9BEG4	
St6GalNAc-VI	<i>Bos taurus</i>	n.d.	AJ620949	CAF06586.1		
CDS4	<i>Branchiostoma floridae</i>	n.d.	AF391289	AAM18873.1	Q8T771	
polysialyltransferase (PST) (fragment) ST8Sia IV	<i>Cercopithecus aethiops</i>	2.4.99.-	AF210729	AAF17105.1	Q9TT09	
polysialyltransferase (STX) (fragment) ST8Sia II	<i>Cercopithecus aethiops</i>	2.4.99.-	AF210318	AAF17104.1	Q9TT10	
α -2,3-sialyltransferase ST3Gal I (Siat4)	<i>Ciona intestinalis</i>	n.d.	AJ626815	CAF25173.1		
α -2,3-sialyltransferase ST3Gal I (Siat4)	<i>Ciona savignyi</i>	n.d.	AJ626814	CAF25172.1		
α -2,8-polysialyltransferase ST8Sia IV	<i>Cricetulus griseus</i>	2.4.99.-	Z46801	AAE28634 CAA86822.1	Q64690	
Gal β -1,3/4-GlcNAc α -2,3-sialyltransferase St3Gal I	<i>Cricetulus griseus</i>	n.d.	AY266675	AAP22942.1	Q80WL0	
Gal β -1,3/4-GlcNAc α -2,3-sialyltransferase St3Gal II (fragment)	<i>Cricetulus griseus</i>	n.d.	AY266676	AAP22943.1	Q80WK9	
α -2,3-sialyltransferase ST3Gal I (Siat4)	<i>Danio rerio</i>	n.d.	AJ783740	CAH04017.1		
α -2,3-sialyltransferase ST3Gal II (Siat5)	<i>Danio rerio</i>	n.d.	AJ783741	CAH04018.1		

FIGURE 1B

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
α -2,3-sialyltransferase ST3Gal III (Siat6)	<i>Danio rerio</i>	n.d.	AJ626821	CAF25179.1		
α -2,3-sialyltransferase ST3Gal IV (Siat4c)	<i>Danio rerio</i>	n.d.	AJ744809	CAG32845.1		
α -2,3-sialyltransferase ST3Gal V-r (Siat5-related)	<i>Danio rerio</i>	n.d.	AJ783742	CAH04019.1		
α -2,6-sialyltransferase ST6Gal I (Siat1)	<i>Danio rerio</i>	n.d.	AJ744801	CAG32837.1		
α -2,6-sialyltransferase ST6GalNAc II (Siat7B)	<i>Danio rerio</i>	n.d.	AJ634459	CAG25680.1		
α -2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	<i>Danio rerio</i>	n.d.	AJ646874	CAG26703.1		
α -2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	<i>Danio rerio</i>	n.d.	AJ646883	CAG26712.1		
α -2,8-sialyltransferase ST8Sia I (Siat 8A) (fragment)	<i>Danio rerio</i>	n.d.	AJ715535	CAG29374.1		
α -2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)	<i>Danio rerio</i>	n.d.	AJ715543	CAG29382.1		
α -2,8-sialyltransferase ST8Sia IV (Siat 8D) (fragment)	<i>Danio rerio</i>	n.d.	AJ715545	CAG29384.1		
α -2,8-sialyltransferase ST8Sia V (Siat 8E) (fragment)	<i>Danio rerio</i>	n.d.	AJ715546	CAG29385.1		
α -2,8-sialyltransferase ST8Sia VI (Siat 8F) (fragment)	<i>Danio rerio</i>	n.d.	AJ715551	CAG29390.1		
β -galactosamide α -2,6-sialyltransferase II (ST6Gal II)	<i>Danio rerio</i>	n.d.	AJ627627	CAF29495.1		
<i>N</i> -glycan α -2,8-sialyltransferase	<i>Danio rerio</i>	n.d.	BC050483 AY055462 NM_153662	AAH50483.1 AAL17875.1 NP_705948.1	Q7ZU51 Q8QH83	
ST3Gal III-related (siat6r)	<i>Danio rerio</i>	n.d.	BC053179 AJ626820 NM_200355	AAH53179.1 CAF25178.1 NP_956649.1	Q7T3B9	
St3Gal-V	<i>Danio rerio</i>	n.d.	AJ619960	CAF04061.1		
st6GalNAc-VI	<i>Danio rerio</i>	n.d.	BC060932 AJ620947	AAH60932.1 CAF06584.1		
α -2,6-sialyltransferase (CG4871) ST6Gal I	<i>Drosophila melanogaster</i>	2.4.99.1	AE003465 AF218237 AF397532 AE003465 NM_079129 NM_166684	AAF47256.1 AAG13185.1 AAK92126.1 AAM70791.1 NP_523853.1 NP_726474.1	Q9GU23 Q9W121	
α -2,3-sialyltransferase (ST3Gal-VI)	<i>Gallus gallus</i>	n.d.	AJ585767 AJ627204	CAE51391.1 CAF25503.1		
α -2,3-sialyltransferase ST3Gal I	<i>Gallus gallus</i>	2.4.99.4	X80503 NM_205217	CAA56666.1 NP_990548.1	Q11200	
α -2,3-sialyltransferase ST3Gal IV (fragment)	<i>Gallus gallus</i>	2.4.99.-	AF035250	AAC14163.1	O73724	
α -2,3-sialyltransferase (ST3GAL-II)	<i>Gallus gallus</i>	n.d.	AJ585761	CAE51385.2		
α -2,6-sialyltransferase (Siat7b)	<i>Gallus gallus</i>	n.d.	AJ620653	CAF05852.1		
α -2,6-sialyltransferase ST6Gal I	<i>Gallus gallus</i>	2.4.99.1	X755558 NM_205241	CAA53235.1 NP_990572.1	Q92182	
α -2,6-sialyltransferase	<i>Gallus gallus</i>	2.4.99.3	-	AAE68028.1	Q92183	

FIGURE 1C

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
ST6GalNAc I				- X74946 NM_205240	AAE68029.1 CAA52902.1 NP_990571.1		
α -2,6-sialyltransferase ST6GalNAc II	<i>Gallus gallus</i>		2.4.99.-	X77775 NM_205233	AAE68030.1 CAA54813.1 NP_990564.1	Q92184	
α -2,6-sialyltransferase ST6GalNAc III (SIAT7C) (fragment)	<i>Gallus gallus</i>		n.d.	AJ634455	CAG25677.1		
α -2,6-sialyltransferase ST6GalNAc V (SIAT7E) (fragment)	<i>Gallus gallus</i>		n.d.	AJ646877	CAG26706.1		
α -2,8-sialyltransferase (GD3 Synthase) ST8Sia	<i>Gallus gallus</i>		2.4.99.-	U73176	AAC28888.1	P79783	
α -2,8-sialyltransferase (SIAT8B)	<i>Gallus gallus</i>		n.d.	AJ699419	CAG27881.1		
α -2,8-sialyltransferase (SIAT8C)	<i>Gallus gallus</i>		n.d.	AJ699420	CAG27882.1		
α -2,8-sialyltransferase (SIAT8F)	<i>Gallus gallus</i>		n.d.	AJ699424	CAG27886.1		
α -2,8-sialyltransferase ST8Sia α -V (SIAT8C)	<i>Gallus gallus</i>		n.d.	AJ704564	CAG28697.1		
β -galactosamide α -2,6-sialyltransferase II (ST6Gal II)	<i>Gallus gallus</i>		n.d.	AJ627629	CAF29497.1		
GM3 synthase (SIAT9)	<i>Gallus gallus</i>		2.4.99.9	AY515255	AAS83519.1		
polysialyltransferase ST8Sia IV	<i>Gallus gallus</i>		2.4.99.-	AF008194	AAB95120.1	O42399	
α -2,3-sialyltransferase ST3Gal I	<i>Homo sapiens</i>		2.4.99.4	L29555 AF059321 L13972 AF155238 AF186191 BC018357 NM_003033 NM_173344	AAA36612.1 AAC17874.1 AAC37574.1 AAD39238.1 AAG29876.1 AAH18357.1 NP_003024.1 NP_775479.1	Q11201 O60677 Q9UN51	
α -2,3-sialyltransferase ST3Gal II	<i>Homo sapiens</i>		2.4.99.4	U63090 BC036777 X96667 NM_006927	AAB40389.1 AAH36777.1 CAA65447.1 NP_008858.1	Q16842 O00654	
α -2,3-sialyltransferase ST3Gal III (SiaT6)	<i>Homo sapiens</i>		2.4.99.6	L23768 BC050380 AF425851 AF425852 AF425853 AF425854 AF425855 AF425856 AF425857 AF425858 AF425859 AF425860 AF425861 AF425862 AF425863 AF425864 AF425865 AF425866 AF425867 AY167992 AY167993 AY167994	AAA35778.1 AAH50380.1 AAO13859.1 AAO13860.1 AAO13861.1 AAO13862.1 AAO13863.1 AAO13864.1 AAO13865.1 AAO13866.1 AAO13867.1 AAO13868.1 AAO13869.1 AAO13870.1 AAO13871.1 AAO13872.1 AAO13873.1 AAO13874.1 AAO13875.1 AAO38806.1 AAO38807.1 AAO38808.1	Q11203 Q86UR6 Q86UR7 Q86UR8 Q86UR9 Q86US0 Q86US1 Q86US2 Q8IX43 Q8IX44 Q8IX45 Q8IX46 Q8IX47 Q8IX48 Q8IX49 Q8IX50 Q8IX51 Q8IX52 Q8IX53 Q8IX54 Q8IX55 Q8IX56	

FIGURE 1D

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
				AY167995 AY167996 AY167997 AY167998 NM_006279 NM_174964 NM_174965 NM_174966 NM_174967 NM_174969 NM_174970 NM_174972	AAO38809.1 AAO38810.1 AAO38811.1 AAO38812.1 NP_006270.1 NP_777624.1 NP_777625.1 NP_777626.1 NP_777627.1 NP_777629.1 NP_777630.1 NP_777632.1	Q8IX57 Q8IX58	
α -2,3-sialyltransferase ST3Gal IV		<i>Homo sapiens</i>	2.4.99.-	L23767 AF035249 BC010645 AY040826 AF516602 AF516603 AF516604 AF525084 X74570 CR456858 NM_006278	AAA16460.1 AAC14162.1 AAH10645.1 AAK93790.1 AAM66431.1 AAM66432.1 AAM66433.1 AAM81378.1 CAA52662.1 CAG33139.1 NP_006269.1	Q11206 O60497 Q96QQ9 Q8N6A6 Q8N6A7 Q8NFD3 Q8NFG7	
α -2,3-sialyltransferase ST3Gal VI		<i>Homo sapiens</i>	2.4.99.4	AF119391 BC023312 AB022918 AX877828 AX886023 NM_006100	AAD39131.1 AAH23312.1 BAA77609.1 CAE89895.1 CAF00161.1 NP_006091.1	Q9Y274	
α -2,6-sialyltransferase (ST6Gal II ; KIAA1877)		<i>Homo sapiens</i>	n.d.	BC008680 AB058780 AB059555 AJ512141 AX795193 AX795193 NM_032528	AAH08680.1 BAB47506.1 BAC24793.1 CAD54408.1 CAE48260.1 CAE48261.1 NP_115917.1	Q86Y44 Q8IUG7 Q96HE4 Q96JF0	
α -2,6-sialyltransferase (ST6GALNAC III)		<i>Homo sapiens</i>	n.d.	BC059363 AY358540 AK091215 AJ507291 NM_152996	AAH59363.1 AAQ88904.1 BAC03611.1 CAD45371.1 NP_694541.1	Q8N259 Q8NDV1	
α -2,6-sialyltransferase (ST6GalNAc V)		<i>Homo sapiens</i>	n.d.	BC001201 AK056241 AL035409 AJ507292 NM_030965	AAH01201.1 BAB71127.1 CAB72344.1 CAD45372.1 NP_112227.1	Q9BVH7	
α -2,6-sialyltransferase (SThM) ST6GalNAc II		<i>Homo sapiens</i>	2.4.99.-	U14550 BC040455 AJ251053 NM_006456	AAA52228.1 AAH40455.1 CAB61434.1 NP_006447.1	Q9UJ37 Q12971	
α -2,6-sialyltransferase ST6Gal I		<i>Homo sapiens</i>	2.4.99.1	BC031476 BC040009 A17362 A23699 X17247 X54363 X62822 NM_003032 NM_173216	AAH31476.1 AAH40009.1 CAA01327.1 CAA01686.1 CAA35111.1 CAA38246.1 CAA44634.1 NP_003023.1 NP_775323.1	P15907	
α -2,6-sialyltransferase ST6GalNAc I		<i>Homo sapiens</i>	2.4.99.3	BC022462 AY096001 AY358918 AK000113 Y11339	AAH22462.1 AAM22800.1 AAQ89277.1 BAA90953.1 CAA72179.2	Q8TBJ6 Q9NSC7 Q9NXQ7	

FIGURE 1E

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
α -2,8-polysialyltransferase ST8Sia IV	<i>Homo sapiens</i>		2.4.99.-	NM_018414 L41680 BC027866 BC053657 NM_005668	NP_060884.1 AAC41775.1 AAH27866.1 AAH53657.1 NP_005659.1	Q8N1F4 Q92187 Q92693	
α -2,8-sialyltransferase (GD3 synthase) ST8Sia	<i>Homo sapiens</i>		2.4.99.8	L32867 L43494 BC046158 AY569975 D26360 X77922 NM_003034	AAA62366.1 AAC37586.1 AAH46158.1 AAQ53140.1 AAS75783.1 BAA05391.1 CAA54891.1 NP_003025.1	Q86X71 Q92185 Q93064	
α -2,8-sialyltransferase ST8Sia II	<i>Homo sapiens</i>		2.4.99.-	L29556 U82762 U33551 BC069584 NM_006011	AAA36613.1 AAB51242.1 AAC24458.1 AAH69584.1 NP_006002.1	Q92186 Q92470 Q92746	
α -2,8-sialyltransferase ST8Sia III	<i>Homo sapiens</i>		2.4.99.-	AF004668 AF003092 NM_015879	AAB87642.1 AAC15901.2 NP_056963.1	O43173 Q9NS41	
α -2,8-sialyltransferase ST8Sia V	<i>Homo sapiens</i>		2.4.99.-	U91641 CR457037 NM_013305	AAC51727.1 CAG33318.1 NP_037437.1	O15466	
ENSP00000020221 (fragment)			n.d.	AC023295	-		
lactosylceramide α -2,3-sialyltransferase (ST3Gal V)	<i>Homo sapiens</i>		2.4.99.9	AF105026 AF119415 BC065936 AY152815 AAP65066 AY359105 AB018356 AX876536 NM_003896	AAD14634.1 AAF66146.1 AAH65936.1 AAO16866.1 AAP65066.1 AAQ89463.1 BAA33950.1 CAE89320.1 NP_003887.2	Q9UNP4 O94902	
<i>N</i> -acetylgalactosaminide α -2,6-sialyltransferase (ST6GalNAc VI)	<i>Homo sapiens</i>		2.4.99.-	BC006564 BC007802 BC016299 AY358672 AB035173 AK023900 AJ507293 AX880950 CR457318 NM_013443	AAH06564.1 AAH07802.1 AAH16299.1 AAQ89035.1 BAA87035.1 BAB14715.1 CAD45373.1 CAE91145.1 CAG33599.1 NP_038471.2	Q969X2 Q9H8A2 Q9ULB8	
<i>N</i> -acetylgalactosaminide α -2,6-sialyltransferase IV (ST6GalNAc IV)	<i>Homo sapiens</i>		2.4.99.-	AF127142 BC036705 - AB035172 AK000600 Y17461 AJ271734 AX061620 AX068265 AX969252 NM_014403 NM_175039	AAF00102.1 AAH36705.1 AAP63349.1 BAA87034.1 BAA91281.1 CAB44354.1 CAC07404.1 CAC24981.1 CAC27250.1 CAF14360.1 NP_055218.3 NP_778204.1	Q9H4F1 Q9NWU6 Q9UKU1 Q9ULB9 Q9Y3G3 Q9Y3G4	
ST8SIA-VI (fragment)	<i>Homo sapiens</i>		n.d.	AJ621583 XM_291725	CAF21722.1 XP_291725.2		
unnamed protein product	<i>Homo sapiens</i>		n.d.	AK021929 AX881696	BAB13940.1 CAE91353.1	Q9HAA9	
Gal β -1,3/4-GlcNAc α -	<i>Mesocricetus</i>		2.4.99.6	AJ245699	CAB53394.1	Q9QXF6	

FIGURE 1F

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
2,3-sialyltransferase (ST3Gal III)		<i>auratus</i>					
Gal β -1,3/4-GlcNAc α -2,3-sialyltransferase (ST3Gal IV)		<i>Mesocricetus auratus</i>	2.4.99.6	AJ245700	CAB53395.1	Q9QXF5	
GD3 synthase (fragment) ST8Sia I		<i>Mesocricetus auratus</i>	n.d.	AF141657	AAD33879.1	Q9WUL1	
polysialyltransferase (ST8Sia IV)		<i>Mesocricetus auratus</i>	2.4.99.-	AJ245701	CAB53396.1	Q9QXF4	
α -2,3-sialyltransferase ST3Gal I	<i>St3gal1</i>	<i>Mus musculus</i>	2.4.99.4	AF214028 AK031344 AK078469 X73523 NM_009177	AAF60973.1 BAC27356.1 BAC37290.1 CAA51919.1 NP_033203.1	P54751 Q11202 Q9JL30	
α -2,3-sialyltransferase ST3Gal II	<i>St3gal2</i>	<i>Mus musculus</i>	2.4.99.4	BC015264 BC066064 AK034554 AK034863 AK053827 X76989 NM_009179 NM_178048	AAH15264.1 AAH66064.1 BAC28752.1 BAC28859.1 BAC35543.1 CAA54294.1 NP_033205.1 NP_835149.1	Q11204 Q8BPL0 Q8BSA0 Q8BSE9 Q91WH6	
α -2,3-sialyltransferase ST3Gal III	<i>St3gal3</i>	<i>Mus musculus</i>	2.4.99.-	BC006710 AK005053 AK013016 X84234 NM_009176	AAH06710.1 BAB23779.1 BAB28598.1 CAA59013.1 NP_033202.2	P97325 Q922X5 Q9CZ48 Q9DBB6	
α -2,3-sialyltransferase ST3Gal IV	<i>St3gal4</i>	<i>Mus musculus</i>	2.4.99.4	BC011121 BC050773 D28941 AK008543 AB061305 X95809 NM_009178	AAH11121.1 AAH50773.1 BAA06068.1 BAB25732.1 BAB47508.1 CAA65076.1 NP_033204.2	P97354 Q61325 Q91Y74 Q921R5 Q9CVE8	
α -2,3-sialyltransferase ST3Gal VI	<i>St3gal6</i>	<i>Mus musculus</i>	2.4.99.4	AF119390 BC052338 AB063326 AK033562 AK041173 NM_018784	AAD39130.1 AAH52338.1 BAH79494.1 BAC28360.1 BAC30851.1 NP_061254	Q80UR7 Q8BLV1 Q8VIB3 Q9WVG2	
α -2,6-sialyltransferase ST6GalNAc II	<i>St6galnac2</i>	<i>Mus musculus</i>	2.4.99.-	NM_009180	6677963 BC010208 AB027198 AK004613 X93999 X94000 NM_009180	P70277 Q9DC24 Q9JJM5 AAH10208.1 BAB00637.1 BAB23410.1 CAA63821.1 CAA63822.1 NP_033206.2	
α -2,6-sialyltransferase ST6Gal I	<i>St6gal1</i>	<i>Mus musculus</i>	2.4.99.1	-	AAE68031.1 BC027833 D16106 AK034768 AK084124 NM_145933	Q8BM62 Q8K1L1 AAH27833.1 BAA03680.1 BAC28828.1 BAC39120.1 NP_666045.1	Q64685
α -2,6-sialyltransferase ST6Gal II	<i>St6gal2</i>	<i>Mus musculus</i>	n.d.		AK082566 AB095093 AK129462 NM_172829	BAC38534.1 BAC87752.1 BAC98272.1 NP_766417.1	Q8BUU4
α -2,6-sialyltransferase ST6GalNAc I	<i>St6galnac1</i>	<i>Mus musculus</i>	2.4.99.3	Y11274 NM_011371	CAA72137.1 NP_035501.1	Q9QZ39 Q9JJP5	
α -2,6-sialyltransferase ST6GalNAc III	<i>St6galnac3</i>	<i>Mus musculus</i>	n.d.	BC058387 AK034804 Y11342 Y11343	AAH58387.1 BAC28836.1 CAA72181.2 CAB95031.1	Q9VUV2 Q9JHP5	

FIGURE 1G

Protein	Organism		EC#	GenBank / GenPept		SwissProt	PDB / 3D
				NM_011372	NP_035502		
α -2,6-sialyltransferase ST6GalNAc IV	<i>St6galnac4</i>	<i>Mus musculus</i>	2.4.99.7	BC056451 AK085730 AJ007310 Y15779 Y15780 Y19055 Y19057 NM_011373	AAH56451.1 BAC39523.1 CAA07446.1 CAB43507.1 CAB43514.1 CAB93946.1 CAB93948.1 NP_035503.1	Q8C3J2 Q9JHP2 Q9R2B6 O88725 Q9JHP0 Q9QUP9 Q9R2B5	
α -2,8-sialyltransferase (GD3 synthase) ST8Sia I	<i>St8sia1</i>	<i>Mus musculus</i>	2.4.99.8	L38677 BC024821 AK046188 AK052444 X84235 AJ401102 NM_011374	AAA91869.1 AAH24821.1 BAC32625.1 BAC34994.1 CAA59014.1 CAC20706.1 NP_035504.1	Q64468 Q64687 Q8BL76 Q8BW10 Q8K1C1 Q9EPK0	
α -2,8-sialyltransferase (ST8Sia VI)	<i>St8sia6</i>	<i>Mus musculus</i>	n.d.	AB059554 AK085105 NM_145838	BAC01265.1 BAC39367.1 NP_665837.1	Q8BI43 Q8K4T1	
α -2,8-sialyltransferase ST8Sia II	<i>St8sia2</i>	<i>Mus musculus</i>	2.4.99.-	X83562 X99646 X99647 X99648 X99649 X99650 X99651 NM_009181	CAA58548.1 CAA67965.1 CAA67965.1 CAA67965.1 CAA67965.1 CAA67965.1 CAA67965.1 NP_033207.1	O35696	
α -2,8-sialyltransferase ST8Sia IV	<i>St8sia4</i>	<i>Mus musculus</i>	2.4.99.8	BC060112 AK003690 AK041723 AJ223956 X86000 Y09484 NM_009183	AAH60112.1 BAB22941.1 BAC31044.1 CAA11685.1 CAA59992.1 CAA70692.1 NP_033209.1	Q64692 Q8BY70	
α -2,8-sialyltransferase ST8Sia V	<i>St8sia5</i>	<i>Mus musculus</i>	2.4.99.-	BC034855 AK078670 X98014 X98014 X98014 NM_013666	AAH34855.1 BAC37354.1 CAA66642.1 CAA66643.1 CAA66644.1 NP_038694.1	P70126 P70127 P70128 Q8BJW0 Q8JZQ3	
α -2,8-sialyltransferase ST8Sia III	<i>St8sia3</i>	<i>Mus musculus</i>	2.4.99.-	BC075645 AK015874 X80502 NM_009182	AAH75645.1 BAB30012.1 CAA56665.1 NP_033208.1	Q64689 Q9CUJ6	
GD1 synthase (ST6GalNAc V)	<i>St6galnac5</i>	<i>Mus musculus</i>	n.d.	BC055737 AB030836 AB028840 AK034387 AK038434 AK042683 NM_012028	AAH55737.1 BAA85747.1 BAA89292.1 BAC28693.1 BAC29997.1 BAC31331.1 NP_036158.2	Q8CAM7 Q8CBX1 Q9QYJ1 Q9R0K6	
GM3 synthase (α -2,3-sialyltransferase) ST3Gal V	<i>St3gal5</i>	<i>Mus musculus</i>	2.4.99.9	AF119416 AB018048 AB013302 AK012961 Y15003 NM_011375	AAF66147.1 AAP65063.1 BAA33491.1 BAA76467.1 BAB28571.1 CAA75235.1 NP_035505.1	O88829 Q9CZ65 Q9QWF9	
<i>N</i> -acetylgalactosaminide α -2,6-sialyltransferase (ST6GalNAc VI)	<i>St6galnac6</i>	<i>Mus musculus</i>	2.4.99.-	BC036985 AB035174 AB035123 AK030648	AAH36985.1 BAA87036.1 BAA95940.1 BAC27064.1	Q8CDC3 Q8JZW3 Q9JM95 Q9R0G9	

FIGURE 1H

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
			NM_016973	NP_058669.1		
M138L	<i>Myxoma virus</i>	n.d.	U46578 AF170726 NC_001132	AAD00069.1 AAE61323.1 AAE61326.1 AAF15026.1 NP_051852.1		
α -2,3-sialyltransferase (St3Gal-I)	<i>Oncorhynchus mykiss</i>	n.d.	AJ585760	CAE51384.1		
α -2,6-sialyltransferase (Siat1)	<i>Oncorhynchus mykiss</i>	n.d.	AJ620649	CAF05848.1		
α -2,8-polysialyltransferase IV (ST8Sia IV)	<i>Oncorhynchus mykiss</i>	n.d.	AB094402	BAC77411.1	Q7T2X5	
GalNAc α -2,6-sialyltransferase (RtST6GalNAc)	<i>Oncorhynchus mykiss</i>	n.d.	AB097943	BAC77520.1	Q7T2X4	
α -2,3-sialyltransferase ST3Gal IV	<i>Oryctolagus cuniculus</i>	2.4.99.-	AF121967	AAF28871.1	Q9N257	
OJ1217_F02.7	<i>Oryza sativa (japonica cultivar-group)</i>	n.d.	AP004084	BAD07616.1		
OSJNBa0043L24.2 or OSJNBb0002J11.9	<i>Oryza sativa (japonica cultivar-group)</i>	n.d.	AL731626 AL662969	CAD41185.1 CAE04714.1		
P0683f02.18 or P0489B03.1	<i>Oryza sativa (japonica cultivar-group)</i>	n.d.	AP003289 AP003794	BAB63715.1 BAB90552.1		
α -2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	<i>Oryzias latipes</i>	n.d.	AJ646876	CAG26705.1		
α -2,3-sialyltransferase ST3Gal I (Siat4)	<i>Pan troglodytes</i>	n.d.	AJ744803	CAG32839.1		
α -2,3-sialyltransferase ST3Gal II (Siat5)	<i>Pan troglodytes</i>	n.d.	AJ744804	CAG32840.1		
α -2,3-sialyltransferase ST3Gal III (Siat6)	<i>Pan troglodytes</i>	n.d.	AJ626819	CAF25177.1		
α -2,3-sialyltransferase ST3Gal IV (Siat4c)	<i>Pan troglodytes</i>	n.d.	AJ626824	CAF25182.1		
α -2,3-sialyltransferase ST3Gal VI (Siat10)	<i>Pan troglodytes</i>	n.d.	AJ744808	CAG32844.1		
α -2,6-sialyltransferase (Sia7A)	<i>Pan troglodytes</i>	n.d.	AJ748740	CAG38615.1		
α -2,6-sialyltransferase (Sia7B)	<i>Pan troglodytes</i>	n.d.	AJ748741	CAG38616.1		
α -2,6-sialyltransferase ST6GalNAc III (Siat7C)	<i>Pan troglodytes</i>	n.d.	AJ634454	CAG25676.1		
α -2,6-sialyltransferase ST6GalNAc IV (Siat7D) (fragment)	<i>Pan troglodytes</i>	n.d.	AJ646870	CAG26699.1		
α -2,6-sialyltransferase ST6GalNAc V (Siat7E)	<i>Pan troglodytes</i>	n.d.	AJ646875	CAG26704.1		
α -2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	<i>Pan troglodytes</i>	n.d.	AJ646882	CAG26711.1		
α -2,8-sialyltransferase 8A (Siat8A)	<i>Pan troglodytes</i>	2.4.99.8	AJ697658	CAG26896.1		
α -2,8-sialyltransferase 8B (Siat8B)	<i>Pan troglodytes</i>	n.d.	AJ697659	CAG26897.1		
α -2,8-sialyltransferase 8C (Siat8C)	<i>Pan troglodytes</i>	n.d.	AJ697660	CAG26898.1		
α -2,8-sialyltransferase 8D (Siat8D)	<i>Pan troglodytes</i>	n.d.	AJ697661	CAG26899.1		
α -2,8-sialyltransferase	<i>Pan troglodytes</i>	n.d.	AJ697662	CAG26900.1		

FIGURE 11

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
8E (Siat8E)						
α -2,8-sialyltransferase	<i>Pan troglodytes</i>	n.d.	AJ697663	CAG26901.1		
8F (Siat8F)						
β -galactosamide α -2,6-sialyltransferase I (ST6Gal I; Siat1)	<i>Pan troglodytes</i>	2.4.99.1	AJ627624	CAF29492.1		
β -galactosamide α -2,6-sialyltransferase II (ST6Gal II)	<i>Pan troglodytes</i>	n.d.	AJ627625	CAF29493.1		
GM3 synthase ST3Gal V (Siat9)	<i>Pan troglodytes</i>	n.d.	AJ744807	CAG32843.1		
S138L	<i>Rabbit fibroma virus Kasza</i>	n.d.	NC_001266	NP_052025		
α -2,3-sialyltransferase ST3Gal III	<i>Rattus norvegicus</i>	2.4.99.6	M97754 NM_031697	AAA42146.1 NP_113885.1	Q02734	
α -2,3-sialyltransferase ST3Gal IV (Siat4c)	<i>Rattus norvegicus</i>	n.d.	AJ626825	CAF25183.1		
α -2,3-sialyltransferase ST3Gal VI	<i>Rattus norvegicus</i>	n.d.	AJ626743	CAF25053.1		
α -2,6-sialyltransferase ST3Gal II	<i>Rattus norvegicus</i>	2.4.99.-	X76988 NM_031695	CAA54293.1 NP_113883.1	Q11205	
α -2,6-sialyltransferase ST6Gal I	<i>Rattus norvegicus</i>	2.4.99.1	M18769 M83143	AAA41196.1 AAB07233.1	P13721	
α -2,6-sialyltransferase ST6GalNAc I (Siat7A)	<i>Rattus norvegicus</i>	n.d.	AJ634458	CAG25684.1		
α -2,6-sialyltransferase ST6GalNAc II (Siat7B)	<i>Rattus norvegicus</i>	n.d.	AJ634457	CAG25679.1		
α -2,6-sialyltransferase ST6GalNAc III	<i>Rattus norvegicus</i>	2.4.99.-	L29554 BC072501 NM_019123	AAC42086.1 AAH72501.1 NP_061996.1	Q64686	
α -2,6-sialyltransferase ST6GalNAc IV (Siat7D) (fragment)	<i>Rattus norvegicus</i>	n.d.	AJ646871	CAG26700.1		
α -2,6-sialyltransferase ST6GalNAc V (Siat7E)	<i>Rattus norvegicus</i>	n.d.	AJ646872	CAG26701.1		
α -2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	<i>Rattus norvegicus</i>	n.d.	AJ646881	CAG26710.1		
α -2,8-sialyltransferase (GD3 synthase) ST8Sia I	<i>Rattus norvegicus</i>	2.4.99.-	U53883 D45255	AAC27541.1 BAA08213.1	P70554 P97713	
α -2,8-sialyltransferase (SIAT8E)	<i>Rattus norvegicus</i>	n.d.	AJ699422	CAG27884.1		
α -2,8-sialyltransferase (SIAT8F)	<i>Rattus norvegicus</i>	n.d.	AJ699423	CAG27885.1		
α -2,8-sialyltransferase ST8Sia II	<i>Rattus norvegicus</i>	2.4.99.-	L13445 NM_057156	AAA42147.1 NP_476497.1	Q07977 Q64688	
α -2,8-sialyltransferase ST8Sia III	<i>Rattus norvegicus</i>	2.4.99.-	U55938 NM_013029	AAB50061.1 NP_037161.1	P97877	
α -2,8-sialyltransferase ST8Sia IV	<i>Rattus norvegicus</i>	2.4.99.-	U90215	AAB49989.1	O08563	
β -galactosamide α -2,6-sialyltransferase II (ST6Gal II)	<i>Rattus norvegicus</i>	n.d.	AJ627626	CAF29494.1		
GM3 synthase ST3Gal V	<i>Rattus norvegicus</i>	n.d.	AB018049 NM_031337	BAA33492.1 NP_112627.1	O88830	

FIGURE 1J

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
sialyltransferase ST3Gal-I (Siat4A)	<i>Rattus norvegicus</i>	n.d.	AJ748840	CAG4449.1		
α -2,3-sialyltransferase (St3Gal-II)	<i>Silurana tropicalis</i>	n.d.	AJ585763	CAE51387.1		
α -2,6-sialyltransferase (Siat7b)	<i>Silurana tropicalis</i>	n.d.	AJ620650	CAF05849.1		
α -2,6-sialyltransferase (St6galnac)	<i>Strongylocentrotus purpuratus</i>	n.d.	AJ699425	CAG27887.1		
α -2,3-sialyltransferase (ST3GAL-III)	<i>Sus scrofa</i>	n.d.	AJ585765	CAE51389.1		
α -2,3-sialyltransferase (ST3GAL-IV)	<i>Sus scrofa</i>	n.d.	AJ584674	CAE48299.1		
α -2,3-sialyltransferase ST3Gal-I	<i>Sus scrofa</i>	2.4.99.4	M97753	AAA31125.1	Q02745	
α -2,6-sialyltransferase (fragment) ST6Gal I	<i>Sus scrofa</i>	2.4.99.1	AF136746	AAD33059.1	Q9XSG8	
β -galactosamide α -2,6-sialyltransferase (ST6GalNAc-V)	<i>Sus scrofa</i>	n.d.	AJ620948	CAF06585.2		
sialyltransferase (fragment) ST6Gal I	<i>sus scrofa</i>	n.d.	AF041031	AAC15633.1	O62717	
ST6GALNAC-V	<i>Sus scrofa</i>	n.d.	AJ620948	CAF06585.1		
α -2,3-sialyltransferase (Siat5-r)	<i>Takifugu rubripes</i>	n.d.	AJ744805	CAG32841.1		
α -2,3-sialyltransferase ST3Gal I (Siat4)	<i>Takifugu rubripes</i>	n.d.	AJ626816	CAF25174.1		
α -2,3-sialyltransferase ST3Gal II (Siat5) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ626817	CAF25175.1		
α -2,3-sialyltransferase ST3Gal III (Siat6)	<i>Takifugu rubripes</i>	n.d.	AJ626818	CAF25176.1		
α -2,6-sialyltransferase ST6Gal I (Siat1)	<i>Takifugu rubripes</i>	n.d.	AJ744800	CAG32836.1		
α -2,6-sialyltransferase ST6GalNAc II (Siat7B)	<i>Takifugu rubripes</i>	n.d.	AJ634460	CAG25681.1		
α -2,6-sialyltransferase ST6GalNAc II B (Siat7B-related)	<i>Takifugu rubripes</i>	n.d.	AJ634461	CAG25682.1		
α -2,6-sialyltransferase ST6GalNAc III (Siat7C) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ634456	CAG25678.1		
α -2,6-sialyltransferase ST6GalNAc IV (siat7D) (fragment)	<i>Takifugu rubripes</i>	2.4.99.3	Y17466 AJ646869	CAB44338.1 CAG26698.1	Q9W6U6	
α -2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ646873	CAG26702.1		
α -2,6-sialyltransferase ST6GalNAc VI (Siat7F) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ646880	CAG26709.1		
α -2,8-sialyltransferase ST8Sia I (Siat 8A) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ715534	CAG29373.1		
α -2,8-sialyltransferase ST8Sia II (Siat 8B) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ715538	CAG29377.1		
α -2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ715541	CAG29380.1		
α -2,8-sialyltransferase ST8Sia IIIr (Siat 8Cr)	<i>Takifugu rubripes</i>	n.d.	AJ715542	CAG29381.1		
α -2,8-sialyltransferase ST8Sia V (Siat 8E)	<i>Takifugu rubripes</i>	n.d.	AJ715547	CAG29386.1		

FIGURE 1K

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
(fragment)						
α -2,8-sialyltransferase ST8Sia VI (Siat 8F) (fragment)	<i>Takifugu rubripes</i>	n.d.	AJ715549	CAG29388.1		
α -2,8-sialyltransferase ST8Sia Vlr (Siat 8Fr)	<i>Takifugu rubripes</i>	n.d.	AJ715550	CAG29389.1		
α -2,3-sialyltransferase (Siat5-r)	<i>Tetraodon nigroviridis</i>	n.d.	AJ744806	CAG32842.1		
α -2,3-sialyltransferase ST3Gal I (Siat4)	<i>Tetraodon nigroviridis</i>	n.d.	AJ744802	CAG32838.1		
α -2,3-sialyltransferase ST3Gal III (Siat6)	<i>Tetraodon nigroviridis</i>	n.d.	AJ626822	CAF25180.1		
α -2,6-sialyltransferase ST6GalNAc II (Siat7B)	<i>Tetraodon nigroviridis</i>	n.d.	AJ634462	CAG25683.1		
α -2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	<i>Tetraodon nigroviridis</i>	n.d.	AJ646879	CAG26708.1		
α -2,8-sialyltransferase ST8Sia I (Siat 8A) (fragment)	<i>Tetraodon nigroviridis</i>	n.d.	AJ715536	CAG29375.1		
α -2,8-sialyltransferase ST8Sia II (Siat 8B) (fragment)	<i>Tetraodon nigroviridis</i>	n.d.	AJ715537	CAG29376.1		
α -2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)	<i>Tetraodon nigroviridis</i>	n.d.	AJ715539	CAG29378.1		
α -2,8-sialyltransferase ST8Sia IIIr (Siat 8Cr) (fragment)	<i>Tetraodon nigroviridis</i>	n.d.	AJ715540	CAG29379.1		
α -2,8-sialyltransferase ST8Sia V (Siat 8E) (fragment)	<i>Tetraodon nigroviridis</i>	n.d.	AJ715548	CAG29387.1		
α -2,3-sialyltransferase (St3Gal-II)	<i>Xenopus laevis</i>	n.d.	AJ585762	CAE51386.1		
α -2,3-sialyltransferase (St3Gal-VI)	<i>Xenopus laevis</i>	n.d.	AJ585766	CAE51390.1		
α -2,3-sialyltransferase St3Gal-III (Siat6)	<i>Xenopus laevis</i>	n.d.	AJ585764 AJ626823	CAE51388.1 CAF25181.1		
α -2,8-polysialyltransferase	<i>Xenopus laevis</i>	2.4.99.-	AB007468	BAA32617.1	O93234	
α -2,8-sialyltransferase ST8Sia-I (Siat8A;GD3 synthase)	<i>Xenopus laevis</i>	n.d.	AY272056 AY272057 AJ704562	AAQ16162.1 AAQ16163.1 CAG28695.1		
Unknown (protein for MGC:81265)	<i>Xenopus laevis</i>	n.d.	BC068760	AAH68760.1		
α -2,3-sialyltransferase (3Gal-VI)	<i>Xenopus tropicalis</i>	n.d.	AJ626744	CAF25054.1		
α -2,3-sialyltransferase (Siat4c)	<i>Xenopus tropicalis</i>	n.d.	AJ622908	CAF22058.1		
α -2,6-sialyltransferase ST6GalNAc V (Siat7E) (fragment)	<i>Xenopus tropicalis</i>	n.d.	AJ646878	CAG26707.1		
α -2,8-sialyltransferase ST8Sia III (Siat 8C) (fragment)	<i>Xenopus tropicalis</i>	n.d.	AJ715544	CAG29383.1		
β -galactosamide α -2,6-sialyltransferase II (ST6Gal II)	<i>Xenopus tropicalis</i>	n.d.	AJ627628	CAF29496.1		
sialyltransferase St8Sial	<i>Xenopus tropicalis</i>	n.d.	AY652775	AAT67042		
poly- α -2,8-sialosyl sialyltransferase (NeuS)	<i>Escherichia coli K1</i>	2.4.--	M76370 X60598	AAA24213.1 CAA43053.1	Q57269	
polysialyltransferase	<i>Escherichia coli K92</i>	2.4.--	M88479	AAA24215.1	Q47404	

FIGURE 1L

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
α -2,8 polysialyltransferase SiaD	<i>Neisseria meningitidis</i> B1940	2.4.-.-	M95053 X78068	AAA20478.1 CAA54985.1	Q51281 Q51145	
SynE	<i>Neisseria meningitidis</i> FAM18	n.d.	U75650	AAB53842.1	O06435	
polysialyltransferase (SiaD)(fragment)	<i>Neisseria meningitidis</i> M1019	n.d.	AY234192	AAO85290.1		
SiaD (fragment)	<i>Neisseria meningitidis</i> M209	n.d.	AY281046	AAP34769.1		
SiaD (fragment)	<i>Neisseria meningitidis</i> M3045	n.d.	AY281044	AAP34767.1		
polysialyltransferase (SiaD)(fragment)	<i>Neisseria meningitidis</i> M3315	n.d.	AY234191	AAO85289.1		
SiaD (fragment)	<i>Neisseria meningitidis</i> M3515	n.d.	AY281047	AAP34770.1		
polysialyltransferase (SiaD)(fragment)	<i>Neisseria meningitidis</i> M4211	n.d.	AY234190	AAO85288.1		
SiaD (fragment)	<i>Neisseria meningitidis</i> M4642	n.d.	AY281048	AAP34771.1		
polysialyltransferase (SiaD)(fragment)	<i>Neisseria meningitidis</i> M5177	n.d.	AY234193	AAO85291.1		
SiaD	<i>Neisseria meningitidis</i> M5178	n.d.	AY281043	AAP34766.1		
SiaD (fragment)	<i>Neisseria meningitidis</i> M980	n.d.	AY281045	AAP34768.1		
NMB0067	<i>Neisseria meningitidis</i> MC58	n.d.	NC_003112	NP_273131		
Lst	<i>Aeromonas punctata</i> Sch3	n.d.	AF126256	AAS66624.1		
ORF2	<i>Haemophilus influenzae</i> A2	n.d.	M94855	AAA24979.1		
HI1699	<i>Haemophilus influenzae</i> Rd	n.d.	U32842 NC_000907	AAC23345.1 NP_439841.1	Q48211	
α -2,3-sialyltransferase	<i>Neisseria gonorrhoeae</i> F62	2.4.99.4	U60664	AAC44539.1 AAE67205.1	P72074	
α -2,3-sialyltransferase	<i>Neisseria meningitidis</i> 126E, NRCC 4010	2.4.99.4	U60662	AAC44544.2		
α -2,3-sialyltransferase	<i>Neisseria meningitidis</i> 406Y, NRCC 4030	2.4.99.4	U60661	AAC44543.1		
α -2,3-sialyltransferase (NMB0922)	<i>Neisseria meningitidis</i> MC58	2.4.99.4	U60660 AE002443 NC_003112	AAC44541.1 AAF41330.1 NP_273962.1	P72097	
NMA1118	<i>Neisseria meningitidis</i> Z2491	n.d.	AL162755 NC_003116	CAB84380.1 NP_283887.1	Q9JUV5	
PM0508	<i>Pasteurella multocida</i> PM70	n.d.	AE006086 NC_002663	AAK02592.1 NP_245445.1	Q9CNC4	
WaaH	<i>Salmonella enterica</i> SARB25	n.d.	AF519787	AAM82550.1	Q8KS93	
WaaH	<i>Salmonella enterica</i> SARB3	n.d.	AF519788	AAM82551.1	Q8KS92	
WaaH	<i>Salmonella enterica</i> SARB39	n.d.	AF519789	AAM82552.1		
WaaH	<i>Salmonella enterica</i> SARB53	n.d.	AF519790	AAM82553.1		
WaaH	<i>Salmonella enterica</i> SARB57	n.d.	AF519791	AAM82554.1	Q8KS91	
WaaH	<i>Salmonella enterica</i> SARB71	n.d.	AF519793	AAM82556.1	Q8KS89	
WaaH	<i>Salmonella enterica</i>	n.d.	AF519792	AAM82555.1	Q8KS90	

FIGURE 1M

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
	SARB8					
WaaH	<i>Salmonella enterica</i> SARC10V	n.d.	AF519779	AAM88840.1	Q8KS99	
WaaH (fragment)	<i>Salmonella enterica</i> SARC12	n.d.	AF519781	AAM88842.1		
WaaH (fragment)	<i>Salmonella enterica</i> SARC13I	n.d.	AF519782	AAM88843.1	Q8KS98	
WaaH (fragment)	<i>Salmonella enterica</i> SARC14I	n.d.	AF519783	AAM88844.1	Q8KS97	
WaaH	<i>Salmonella enterica</i> SARC15II	n.d.	AF519784	AAM88845.1	Q8KS96	
WaaH	<i>Salmonella enterica</i> SARC16II	n.d.	AF519785	AAM88846.1	Q8KS95	
WaaH (fragment)	<i>Salmonella enterica</i> SARC3I	n.d.	AF519772	AAM88834.1	Q8KSA4	
WaaH (fragment)	<i>Salmonella enterica</i> SARC4I	n.d.	AF519773	AAM88835.1	Q8KSA3	
WaaH	<i>Salmonella enterica</i> SARC5IIa	n.d.	AF519774	AAM88836.1		
WaaH	<i>Salmonella enterica</i> SARC6IIa	n.d.	AF519775	AAM88837.1	Q8KSA2	
WaaH	<i>Salmonella enterica</i> SARC8	n.d.	AF519777	AAM88838.1	Q8KSA1	
WaaH	<i>Salmonella enterica</i> SARC9V	n.d.	AF519778	AAM88839.1	Q8KSA0	
UDP-glucose : α -1,2-glucosyltransferase (WaaH)	<i>Salmonella enterica</i> subsp. <i>arizonaee</i> SARC 5	2.4.1.-	AF511116	AAM48166.1		
bifunctional α -2,3/-2,8-sialyltransferase (Cst-II)	<i>Campylobacter jejuni</i> ATCC 43449	n.d.	AF401529	AAL06004.1	Q93CZ5	
Cst	<i>Campylobacter jejuni</i> 81-176	n.d.	AF305571	AAL09368.1		
α -2,3-sialyltransferase (Cst-III)	<i>Campylobacter jejuni</i> ATCC 43429	2.4.99.-	AY044156	AAK73183.1		
α -2,3-sialyltransferase (Cst-III)	<i>Campylobacter jejuni</i> ATCC 43430	2.4.99.-	AF400047	AAK85419.1		
α -2,3-sialyltransferase (Cst-II)	<i>Campylobacter jejuni</i> ATCC 43432	2.4.99.-	AF215659	AAG43979.1	Q9F0M9	
α -2,3/8-sialyltransferase (CstII)	<i>Campylobacter jejuni</i> ATCC 43438	n.d.	AF400048	AAK91725.1	Q93MQ0	
α -2,3-sialyltransferase cst-II	<i>Campylobacter jejuni</i> ATCC 43446	2.4.99.-	AF167344	AAF34137.1		
α -2,3-sialyltransferase (Cst-II)	<i>Campylobacter jejuni</i> ATCC 43456	2.4.99.-	AF401528	AAL05990.1	Q93D05	
α -2,3/- α -2,8-sialyltransferase (CstII)	<i>Campylobacter jejuni</i> ATCC 43460	2.4.99.-	AY044868	AAK96001.1	Q938X6	
α -2,3/8-sialyltransferase (Cst-II)	<i>Campylobacter jejuni</i> ATCC 700297	n.d.	AF216647	AAL36462.1		
ORF	<i>Campylobacter jejuni</i> GB11	n.d.	AY422197	AAR82875.1		
α -2,3-sialyltransferase cstIII	<i>Campylobacter jejuni</i> MSC57360	2.4.99.-	AF195055	AAG29922.1		
α -2,3-sialyltransferase cstIII Cj1140	<i>Campylobacter jejuni</i> NCTC 11168	2.4.99.-	AL139077 NC_002163	CAB73395.1 NP_282288.1	Q9PNF4	
α -2,3/ α -2,8-sialyltransferase II (cstII)	<i>Campylobacter jejuni</i> O:10	n.d.	- AX934427	AAO96669.1 CAF04167.1		
α -2,3/ α -2,8-sialyltransferase II (CstII)	<i>Campylobacter jejuni</i> O:19	n.d.	AX934431	CAF04169.1		
α -2,3/ α -2,8-sialyltransferase II (CstII)	<i>Campylobacter jejuni</i> O:36	n.d.	AX934436	CAF04171.1		
α -2,3/ α -2,8-	<i>Campylobacter</i>	n.d.	AX934434	CAF04170.1		

FIGURE 1N

Protein	Organism	EC#	GenBank / GenPept		SwissProt	PDB / 3D
sialyltransferase II (CstII)	<i>jejuni</i> O:4					
α -2,3/ α -2,8-sialyltransferase II (CstII)	<i>Campylobacter jejuni</i> O:41	n.d.	- - AX934429	AAO96670.1 AAT17967.1 CAF04168.1		
α -2,3-sialyltransferase cst-I	<i>Campylobacter jejuni</i> OH4384	2.4.99.-	AF130466	AAF13495.1 AAS36261.1	Q9RGF1	
bifunctional α -2,3/-2,8-sialyltransferase (Cst-II)	<i>Campylobacter jejuni</i> OH4384	2.4.99.-	AF130984 AX934425	AAF31771.1 CAF04166.1	1R07 1R08	C A
HI0352 (fragment)	<i>Haemophilus influenzae</i> Rd	n.d.	U32720 X57315 NC_000907	AAC22013.1 CAA40567.1 NP_438516.1	P24324	
PM1174	<i>Pasteurella multocida</i> PM70	n.d.	AE006157 NC_002663	AAK03258.1 NP_246111.1	Q9CLP3	
Sequence 10 from patent US 6503744	Unknown.	n.d.	-	AAO96672.1		
Sequence 10 from patent US 6699705	Unknown.	n.d.	-	AAT17969.1		
Sequence 12 from patent US 6699705	Unknown.	n.d.	-	AAT17970.1		
Sequence 2 from patent US 6709834	Unknown.	n.d.	-	AAT23232.1		
Sequence 3 from patent US 6503744	Unknown.	n.d.	-	AAO96668.1		
Sequence 3 from patent US 6699705	Unknown.	n.d.	-	AAT17965.1		
Sequence 34 from patent US 6503744	Unknown.	n.d.	-	AAO96684.1		
Sequence 35 from patent US 6503744 (fragment)	Unknown.	n.d.	- -	AAO96685.1 AAS36262.1		
Sequence 48 from patent US 6699705	Unknown.	n.d.	-	AAT17988.1		
Sequence 5 from patent US 6699705	Unknown.	n.d.	-	AAT17966.1		
Sequence 9 from patent US 6503744	Unknown.	n.d.	-	AAO96671.1		